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Voor Tim en Anne-Fleur

Gaan we morgen samen spelen
Gaan we lachen in de zon
Ik zal je zingen van het leven
En hoe dat pas bij jou begon

Acda en de Munnik - Slaapliedje

CONTENTS

CHAPTER 1

General Introduction

ASTHMA

Asthma is a chronic inflammatory disease of the airways characterized by variable airway obstruction in association with increased airway hyperresponsiveness (AHR)[1]. Asthma is a serious global health problem and individuals of all ages are affected by this disease. Within adolescents aged 13-14 years, asthma prevalence world wide is estimated to be 14%[2]. Most asthmatics develop asthma during early childhood, with boys having a higher prevalence compared to girls. However, during puberty a gender switch in asthma prevalence occurs, with females having a higher prevalence than males[3]. After menopause, the gap in asthma prevalence between females and males becomes smaller, however, the female dominance does not fully disappear[4].

Common symptoms of asthma are cough, wheeze and shortness of breath. These symptoms are caused by non-allergic stimuli, like fog, smoke and viral infections, and by exposure to allergens, such as house dust mite, (grass) pollens, fungi or air pollution. Exposure to these allergens results in an uptake by antigen presenting cells (APCs) which are presented throughout the whole respiratory tract[5]. These APCs activate T helper 2 (Th2) cells resulting in the production and secretion of cytokines (i.e. interleukin 3 (IL3), IL4, IL5, IL9, IL13 and granulocyte macrophage colony-stimulating factor (GM-CSF))[1]. These cytokines lead among others to the activation of eosinophil maturation and survival, basophil recruitment and the production of immunoglobuline E (IgE) by B cells[1,5]. After re-exposure to this antigen, the mast cells release numerous inflammatory mediators resulting in an acute inflammatory response. This acute inflammatory response consists of vascular leak, bronchospasm and activation of nociceptive neurons linked to parasympathetic reflexes, resulting in clinical symptoms of asthma including cough, wheeze and shortness of breath[6]. At the same time, some mast cell inflammatory mediators up-regulate expression of adhesion molecules for leucocytes (eosinophils, but also basophils and lymphocytes) on endothelial cells. These leucocytes are drawn to the airways by chemoattractant molecules released during a relatively symptom-free recruitment phase during the acute inflammatory response, where they release cytokines and tissue-damaging proteases which results in a second wave of airway inflammation, the so-called late inflammatory response[6]. The repetition of allergen exposure and the subsequent sequelae of memory T lymphocytes and eosinophils that are maintained by paracrine and autocrine cytokines stimulation, may contribute to airway hyperresponsiveness (AHR) and chronic airway symptoms[6].

Complete control of the disease is the main goal of asthma management and this is achieved among others with medication. Asthma symptoms during an acute attack are treated with short-acting β_2-sympathicomimetics, which act quickly to reverse bronchoconstriction and relief symptoms of asthma. Depending on the severity of asthma, individuals with asthma are additionally being treated with maintenance medication at an early stage, such as

glucocorticosteroids (modulating Th2 cytokines and their associated airway inflammation) which are taken prophylactic to control symptoms of asthma[7]. These inhaled corticosteroids are prescribed either alone or in combination with long-acting sympathicomimetics, depending on the severity of the disease.

Nowadays, asthma is not considered as one single disease, but it encompasses multiple subgroups, or phenotypes[8]. In most asthmatics the inflammatory response as a result of exposure to a certain allergen is marked by the Th2 inflammatory response as described above. Asthma phenotypes that belong to this Th2-associated asthma are among others early onset allergic asthma, nocturnal asthma and exercise-induced asthma[8]. Early onset allergic asthmatics develop their asthma during early life, are atopic (produce IgE when exposed to an allergen) and have mostly other allergic diseases such as rhinitis or eczema and/or a family history of allergic diseases[8]. Asthmatics with nocturnal asthma have specifically symptoms of their asthma during midnight which typically reflects more sever asthma, or asthma without control. This is associated with more severe inflammation at day and night and in some aspects more inflammation during the night, which is then less counterbalanced by the lower autonomous nervous system drive as is present at night[9,10]. Asthmatics with exercise-induced asthma have complaints of their asthma particularly during or specifically after stopping their exercise[8].

Although inhaled corticosteroids are the mainstay in asthma treatment, not all asthmatics develop full asthma control with this treatment. This indicates that not all asthma is associated with a Th2 inflammatory response, the so-called non-Th2-asthma[8]. Although little is known about this non-Th2-asthma subgroup, it meets the criteria for asthma. This group presents itself generally with less airway obstruction and AHR than individuals belonging to the Th2-asthma group. Specific phenotypes of this non-Th2-asthma group include very-late onset asthma, obesity associated asthma and neutrophilic asthma[8].

FACTORS ASSOCIATED WITH THE DEVELOPMENT OF ASTHMA

With regard to asthma development, it is known that many pathways are potentially involved in its etiology. Not only multiple genes belonging to several biological pathways (i.e. genes implicated in Th2 cell differentiation and allergic inflammation (i.e. interleukin (*IL*) 4, interleukin 4 receptor (*IL4R*), *IL13*)[11-14] or airway remodeling (A Disintegrin And Metalloprotease 33 (*ADAM33*)[15-19]), but also environmental factors (i.e. exposure to tobacco smoke[20] or air pollution[21]) and gene-environment interactions are known to be associated with the development of asthma or asthma-related phenotypes. However, these factors

do not fully explain the increasing incidence of asthma that has occurred during the past decennia. Therefore, hypotheses on determinants other than those addressing genetic predisposition, environmental exposures and gene-environment interactions have been put forward.

Historically, asthma has been defined as a psychosomatic disorder. In the first half of the 20th century, leading physicians believed that psychological determinants, and especially the quality of mother-child interaction, played a role in the etiology of asthma. As a reaction to these overhyped claims, and with the emerging understanding of the chronic inflammatory process underlying asthma, researchers almost unanimously discarded the role of psychological factors in asthma development. Psychosocial research towards asthma has since then focused on the importance of the asthma patient's behavior with regard to coping with his or her illness. These studies have resulted in very valuable knowledge with important implications for health care[22-24]. One example is the fact that psychosocial distress or negative affect influence the perception of the seriousness of their asthma which result in an overuse of asthma medication. Knowing that such psychosocial mechanisms may contribute to overuse of medication has implications for educating patients on asthma self-management[23].

In the past decade general practitioners, pulmonologists and respiratory researchers started to reconsider the role of stress in asthma. Developments in the relatively new field of psychoneuroimmunology, linking psychosocial stress, the central nervous system, and alterations in immune and endocrine function, provided plausible biological pathways that pinpoint to a role of stress in the etiology of asthma. These new insights have led to a paradigm shift that reconsiders the role of psychological determinants in the etiology of asthma, and focuses on the overlap between biological determinants and psychosocial factors in trying to understand the etiology of asthma. In contrast to earlier psychoanalytic theories, current theories assume psychosocial stress to confer a risk for the development of asthma, additionally to risk factors such as genetic predisposition, and intermediate asthma phenotypes like sensitization to allergens and AHR.

Indeed, a number of recent cross-sectional and longitudinal studies performed in children, adolescents and adults investigated stress as a cause of asthma. These studies investigated a broad variety of stressors, ranging from exposure to stress experienced by the individual itself (i.e. stressful life events, abuse or job stress) to exposure to stress experienced by others such as the parents (i.e. maternal anxiety or depression or parental difficulties). Results from cross-sectional studies found that exposure to violence, physical/sexual abuse, childhood adversities, and job stress was associated with asthma. Another study showed that exposure to domestic violence of the mother was associated with asthma in her children or young adult[25]. Moreover, exposure to domestic violence in women was also associated with asthma in the women themselves[25,26]. However, no association was found

between exposure to community violence and asthma in children[27]. Other studies found that exposure to physical or sexual abuse was associated with asthma in children and adults[27,28]. Childhood adversities[29] and exposure to job stress[30] were also found to be associated with asthma in adults (Tables 1.1 and 1.2). However, when investigating stressful life events as a sum score of different life events, the association between exposure to stressful life events and asthma is less clear. One cross-sectional study found an association between stressful life events and asthma in adolescents[31], whereas another study did not find such an association in children and adolescents[32]. A close comparison of these studies revealed that all studies investigated different stressful life events which hampers comparison of the results from these studies (Tables 1.1 and 1.2).

Summarizing, results from cross-sectional studies suggest that there indeed exists an association between stress and asthma, but no conclusion can be drawn about the causal direction of this association. Recently, longitudinal studies are performed that studied the role of stress in asthma development. Results from birth-cohort studies showed that exposure to psychosocial stress during early life increased the risk to develop asthma. These studies typically investigated the role of parental stress exposure on asthma development in their child and found that maternal anxiety during pregnancy[33], maternal exposure to the death of a child or spouse during pregnancy[34], parental difficulties measured at 3 wk of age[35,36], long term maternal distress (anxiety or depression)[37], stressful life events during 1 year preceding delivery[38] and chronic intimate partner violence[39,40] were associated with an increased risk to develop asthma in their children (Table 1.1). Next to birth-cohort studies, the role of stress exposure on asthma development was also studied in longitudinal studies performed in children and adults. Results from these studies revealed that exposure to community violence[41] increased the risk of asthma development in children, and exposure to stressful life events[42,43], war-related stress[44], work stress[45] and stress in daily life[46] increased the risk of asthma in adults (Tables 1.1 and 1.2).

Although the evidence is accumulating that psychosocial stress is involved in the etiology of asthma (Tables 1.1 and 1.2), a number of important questions remain and have to be clarified regarding the role of the occurrence versus the impact of the stressors, the timing of the exposure to stress and the modification of the effect of stress by genetic susceptibility. In addition, also the mechanisms via which psychosocial stress leads to asthma development need to be clarified. Stress exposure may exert its influence on many levels, making it difficult to disentangle the different mechanisms via which stress relates to asthma development and severity, especially in cross-sectional studies. Stress effects can be classified into (1) physiological stress effects, (2) behavioural stress effects, and (3) psychological stress effects. Of interest, stress has been proposed to affect asthma via all of these pathways.

TABLE 1.1 | Overview of studies investigating association between psychosocial stress and asthma/asthma development in children and adolescents

Reference	Study design	Stress	Outcome	Main finding(s)
		Maternal anxiety during pregnancy		
Cookson[33]	Prospective birth-cohort study of 5,810 children	Maternal anxiety at 18 and 32 weeks of gestation and 8 months of age	Current asthma at age 7,5 yrs	Maternal anxiety during pregnancy was associated with increased risk of asthma onset during childhood
		Bereavement		
Khashan[34]	Prospective birth-cohort study of 3,290,141 children	Prenatal exposure to maternal bereavement[1]	Asthma from hospitalized patients	Prenatal exposure to maternal bereavement was associated with an increased risk of hospitalization for asthma
		Parental stress/caregiver stress		
		High risk cohort		
Mrazek[35]	Prospective birth-cohort study of 150 genetically predisposed children	Parental difficulties measured at 3 wk of age	Asthma onset at age 3 yrs	Parental difficulties measured at 3 wks of age predicted an increased risk of asthma onset by 3 yr of age
Klinnert[36]	Prospective birth-cohort study of 150 genetically predisposed children	Parental difficulties measured at 3 wk of age	Asthma onset between age 6 – 8 yrs	Parental difficulties measured at 3 wk of age were associated with an increased risk for asthma onset between age 6 – 8 yrs
		Non-high risk cohort		
Kozyrskyj[37]	Prospective birth-cohort study of 13,907 children	Maternal distress[2] during the first year of life and onward	Asthma at age 7 yrs	Long term maternal distress was associated with an increased risk for asthma at age 7 yrs

			Stressful life events	
			High risk cohort	
Klinnert[38]	Prospective birth-cohort study of 150 genetically predisposed children	Stressful life events[3] during 1 year preceding delivery of the child	Asthma onset by age 3 yrs	Stressful life events in absence of sufficient parenting mechanisms increased the risk of asthma onset by age 3 yrs
			Non-high risk cohort	
Cohen[27]	Cross-sectional study of 1,213 children	Major life stressors[4] during the previous year	Current asthma	No association between stressful life events and asthma
Turyk[31]	Cross-sectional study of 1,860 adolescents	Stressful life events[5]	Asthma	Asthma was significantly associated with number of total life events, family and violent events, but not with school events
Kilpelainen[32]	Cross-sectional case-control study of 10,667 adolescents	Stressful life events[6] during their life	Asthma (age of onset)	No association between preceding stressful life events and asthma
			Traumatic events/abuse	
Cohen[27]	Cross-sectional study of 1,213 children	Traumatic events[7] in the previous year	Current asthma	History of physical or sexual abuse was associated with an increased risk for asthma

TABLE 1.1 | Continued

Reference	Study design	Stress	Outcome	Main finding(s)
		Violence		
Subramanian[25]	Cross-sectional study of 92,486 households	Domestic violence of the mother	Asthma prevalence	Domestic violence of the mother was associated with asthma in children Domestic violence of the mother was associated with asthma in adolescents
Cohen[27]	Cross-sectional study of 1,213 children	Community violence in the previous year	Current asthma	No association between exposure to community violence and asthma
Suglia[39]	Prospective birth-cohort study of 3,116 children	Maternal intimate partner violence after birth, 12 and 36 months of age	Asthma at age 36 months	Chronic intimate partner violence (IPV) is associated with an increased risk for asthma at age 3 yrs In an analysis stratified by mother-child activities (MCA), children with mothers exposed to chronic IPV and a low level of MCA had a higher risk to develop asthma compared to those exposed to chronic IPV but with a higher level of MCA
Suglia[40]	Prospective birth-cohort study of 3,116 children	Maternal intimate partner violence after birth, 12 and 36 months of age	Asthma at age 36 months	After adjusting for housing deterioration[8], housing disarray[9] and housing hardship[10] the association between IPV (12 or 36 months) and asthma was no longer significant, however, exposure to IPV during both time points was significantly associated with asthma
Sternthal[41]	Longitudinal multilevel study of 2,071 children aged 0–9 yrs	Life time exposure to community violence	Having ever been diagnosed with asthma	Community violence is associated with an increased asthma risk

[1] Death of spouse or child. [2] Depression or anxiety. [3] FILE = Family Inventory of Life Events (71 life events). [4] I.e. death of a family member and parents' divorce. [5] I.e. family/relationship difficulties, school difficulties and experiencing violent events. [6] I.e. disease or death of a family member and conflict in personal or parental relationships (preceding = asthma after exposure to stressful life events, concomitant = asthma and exposure to stressful life events in the same period, subsequent = asthma before exposure to stressful life events). [7] I.e. abuse, neglect and excessive fear of adults "in charge". [8] I.e., peeling paint, holes in floor and broken windows. [9] I.e. dark, cluttered, crowded or noisy house. [10] I.e. Moving repeatedly and hardships in keeping the house warm.

TABLE 1.2 | Overview of studies on the association between psychosocial stress and asthma/asthma development in adults

Reference	Study design	Stress	Outcome	Main finding(s)
		Stressful life events		
Lietzen[42]	Prospective cohort study of 16,881 adults	Stressful life events[1] between baseline and first follow-up	Incident asthma	Stressful life events were associated with asthma Illness of a family member, marital problems, divorce or separation, and conflicts with a supervisor were associated with asthma
Loerbroks[43]	Prospective study of 4,010 adults	Stressful life events[2] during the last 5 years before the baseline measure	Incident asthma	Breaking off a life partnership was associated with asthma
		Traumatic events/abuse		
Romans[28]	Cross-sectional case-control study of 354 adult women	Childhood sexual abuse Childhood physical abuse Adult sexual abuse Adult physical abuse	Asthma/breathing problems in the previous 12 months	Childhood sexual abuse was associated with asthma Childhood physical abuse was not associated with asthma Adult sexual abuse was not associated with asthma Adult physical abuse was not associated with asthma
Wright[44]	Prospective study of 2,066 adults	War-related stress during the Kuwait invasion	Incidence asthma	War-related stress was associated with asthma incidence
		Childhood adversities (combination stressful life events and traumatic events)		
Scott[29]	Cross-sectional study of 18,303 adults	Childhood adversities[3] before adulthood	Adult onset asthma (>21 yrs)	Experience of two or more childhood adversities were associated with adult-onset asthma Physical abuse, parental death, parental mental disorder, and family violence were associated with adult-onset asthma

TABLE 1.2 | Continued

Reference	Study design	Stress	Outcome	Main finding(s)
		Violence		
Subramanian[25]	Cross-sectional study of 92,486 households	Domestic violence to the mother	Asthma prevalence	Domestic violence to the mother was associated with asthma in this mother Domestic violence to their mother was associated with asthma in young adults
Loxton[26]	Cross-sectional survey of 14,100 adult women	Domestic violence	Asthma	Domestic violence was associated with asthma
		Work-related stress and stress in daily activities		
Eng[30]	Cross-sectional study of 2,903 adults	Job stress	Current asthma Adult-onset asthma	Very or extremely stressful jobs were associated with current asthma and adult-onset asthma
Loerbroks[45]	Prospective study of 3,027 adults	Work stress during current or last job Inability to relax after work during current or last job	Cumulative incidence of asthma	Work stress and inability to relax after work were both associated with asthma
Huovinen[46]	Prospective cohort study of 10,615 adults	Stress in daily activities	Asthma incidence	Stress in daily activities was not associated with asthma incidence

[1] I.e. death of a family member, emotional, physical or sexual violence, severe illness in a family member, death of the mother, a major increase in marital problems, divorce or separation, severe conflicts with a supervisor, severe financial difficulties and death of the father. [2] I.e. unemployment, having broken off a life partnership and death of someone close. [3] Combination of stressful life events and traumatic events.

THE ROLE OF STRESSOR OCCURRENCE VERSUS IMPACT OF STRESSORS

To give a simple definition of "stress" has been proven to be extremely difficult[47]. Stress has been defined as environmental demands that individuals appraise as exceeding their coping abilities[47]. This definition clearly indicates that stress comprises two components: the presence of a stressor (i.e. a demand from an individual's environment) and the subjective experience of stress due to the presence of this stressor (i.e. the appraisal that the stressor exceeds the coping abilities). So far, in studies on the role of stress as an additional determinant to the development of asthma, a broad variety of operationalizations and definitions of psychosocial stress have been used, such as exposure to maternal anxiety during pregnancy, parental difficulties, stressful life events such as illness or death of a close relative, traumatic events such as physical or sexual abuse, violence, childhood adversities, stress in daily life and work-related stress. Thus, some studies used rather objective measures (e.g. death of a close relative), whereas others studied used more subjective judgments of stress (e.g. parental difficulties). Moreover, the same stressor might be perceived differently by different individuals due to stress coping resources. These stress coping resources may be related to an individual's characteristics (e.g. personality) and/or his or her environment (e.g. family functioning). This makes comparison of results from different studies difficult.

THE TIMING OF EXPOSURE TO STRESS

There are two basic conceptual life course models, being "the critical period" model and "the accumulation of risk" model[48], which can be applied to the stress-asthma association. The critical period model underlies the fetal origins of adult disease hypothesis. Especially the perinatal period is an important period in the development of asthma, since both the lung and the immune system develop during this period[49]. Previous studies indeed found that exposure to stress during this period was associated with an increased risk of asthma development. These studies typically investigated asthma development in relation to maternal anxiety during pregnancy[33], maternal exposure to the death of a child or spouse during pregnancy[34], stressful life events during 1 year preceding delivery[38], and parental difficulties measured at 3 wks of age[35,36] (Table 1.1). Thus, results from these studies suggest that the perinatal period is a critical period in the development of asthma and that the critical period model can be applied to the stress-asthma association. However, other studies showed that there are also associations between asthma and stress exposure after this perinatal period. These studies investigated asthma development in relation to long-term maternal distress (anxiety or depression)[37], chronic intimate partner violence[39,40], life time exposure to community violence[41], stressful life events within the previous 5 years

(i.e. illness of a family member, marital problems, divorce or separation, conflicts with a supervisor[42] or break off a life partnership[43]), war-related stress[44] and work related stress[45] (Tables 1.1 and 1.2). Therefore, it remains unclear whether the effect of stress exposure on asthma development is different for the different periods, e.g. whether it is larger when the exposure took place during the perinatal compared to later in life.

The other model is the accumulation of risk model. In this model, the effects of stress accumulate gradually over time. Evidence for this model comes from a previous study that showed that the effect of severe life events, especially in the presence of multiple chronic stressors, increases the likelihood of asthma exacerbation in children aged 6-13 years[50]. Whether this accumulation of risk model also applies for the development of asthma is yet not clarified.

THE EFFECT OF STRESS AND GENETIC SUSCEPTIBILITY

A well-known model in psychiatry is the stress-vulnerability model, which basically states that stress may lead to psychiatric problems if an underlying vulnerability is present. Previous studies performed in children with a familial predisposition for asthma found that exposure to psychosocial stress is associated with an increased risk to develop asthma (Table 1.1). However results from population-based studies are inconsistent. Most studies found an association between stress exposure and asthma development, however, some studies did not (Tables 1.1 and 1.2). This could suggest that a familial predisposition for asthma may modify the association between psychosocial stress exposure and asthma development. However, plausible genes underlying this potentially modifying effect are yet unknown.

THE MECHANISMS VIA WHICH STRESS CONTRIBUTES TO ASTHMA DEVELOPMENT

Physiological stress effects

The effects of stress on physiology are mediated by the fast-acting autonomic nervous system (ANS) (including the sympathetic and parasympathetic branches) and the slower-acting hypothalamus-pituitary-adrenal (HPA) axis, the two axes that link the brain and the immune system.

Autonomic nervous system (ANS)

The ANS controls activities of visceral functions that are not under voluntary control, such as the regulation of the circulation (heart rate and force of contraction), respiration, digestion, body temperature, papillary dilation, and metabolism. The ANS consist of two branches: the

sympathetic nervous system (SNS) and the parasympathetic nervous system (PNS). The SNS activates neurons in the thoracic and lumbar levels of the spinal cord and activation of this system leads to the release of catecholamines (CAs: adrenaline and noradrenaline). The PNS activates neurons in the cranial and sacral levels of the spinal cord and activation of this system leads to the release of acetylcholine. Most organs are innervated by neurons of both systems, however, the function of these systems on these organs is usually opposite (e.g. SNS increases heart rate and induces bronchodilatation, whilst PNS decreases heart rate and induces bronchoconstriction)[51-53].

The Hypothalamus-Pituitary-Adrenal (HPA) axis

Activation of the neurons in the paraventricular nucleus (PVN) of the hypothalamus leads to the secretion of corticotrophin releasing hormone (CRH). This CRH stimulates the anterior pituitary gland resulting in a secretion of adrenocorticotropin hormone (ACTH), which stimulates the adrenal glands to produce cortisol. This whole process from activation of the PVN to the release of cortisol from the adrenal glands takes about 20-30 minutes[53]. In non-stressful situations, the secretion of cortisol follows a circadian rhythm, with a sharp increase prior to awakening, reaching a peak approximately half an hour later (cortisol awakening response (CAR)[54,55]), after which the secretion of cortisol steadily declines and is at its lowest during midnight. Exposure to stress leads to an increase in the activity of the PVN resulting in an increase in cortisol production. Overshooting of the HPA axis is regulated by means of a negative feedback mechanism, meaning that elevated circulating levels of cortisol suppress secretion of both CRH and ACTH, resulting in a decreased secretion of cortisol[53,56]. Cortisol thus plays an important role in the regulation of the basal HPA axis activity and in the duration of the stress response[53]. In the human body, cortisol influences the activity of many systems, including the central nervous system, where it is involved in learning, memory and emotion; the metabolic system, where it regulates glucose storage and utilization; and the immune system, where it regulates the magnitude and duration of the inflammatory responses and the maturation of lymphocytes[53,57].

The link of these systems with asthma development

The effect of stress on asthma development is complex. Acute stress activates both the SNS and HPA axis, leading to an increased secretion of CAs and cortisol. Both CAs and cortisol play a key role in the immune system, where they induce a shift in the Th1/Th2 balance of the peripheral mononuclear blood cells towards a predominant Th2 response[52,53]. Moreover, CRH is also produced in the periphery at the site of inflammation[52,53]. Especially in the early inflammatory response after allergen inhalation, this peripheral CRH plays an important role[52]. Peripheral CRH activates mast cell degradation leading to the release of histamine, causing vasodilatation, edema and bronchoconstriction[52,53]. In addition, histamine inhibits the production of Th1 cytokines[52]. Thereby, activation of peripheral CRH induces acute inflammation and a shift in the Th1/Th2 balance towards a Th2 response[52]. In this way, exposure to acute stress leads to an activation of the stress system and together with the

peripheral production of CRH this facilitates asthma development. However, these effects of stress on asthma development can be antagonized by the direct effect of stress on mast cells. Both cortisol and CAs act directly on mast cells leading to a suppression of the release of histamine and thereby abolishing its inflammatory and bronchoconstrive effect[52]. Moreover, the activation of the SNS by stress leads to bronchodilatation.

Results from previous studies suggests that exposure to stress in a risk factor for asthma development[33-36,38,46]. However, it is yet unclear whether asthma development is associated with high or low cortisol levels. Some studies found that asthmatics had high cortisol levels compared to non-asthmatics[58,59], whereas other studies found no difference[60-64] or even lower[65-67] cortisol levels in asthmatics compared to non-asthmatics. One recent review stated that a blunted HPA axis (characterized by lower morning cortisol levels and flattening of the diurnal slope) is associated with an increased susceptibility to autoimmune and inflammatory diseases[49], and therefore low cortisol levels are associated with asthma. However, how these low cortisol levels lead to the development of asthma is yet not clear.

The role of the autonomic nervous system in relation to asthma has also been studied. That study suggested hat patients with asthma have an increased activity of the parasympathetic and decreased activity of the sympathetic nervous system[68]. However, the role of the ANS in asthma development has to be studied further.

Behavioural stress effects

Stress increases the likelihood of health-risk behaviors such as smoking and behaviors related to overweight (both eating and low activity patterns). Adolescents with a history of stressful events early in life have an increased risk for regular smoking[69], a known risk factor for asthma. Exposure to early life trauma is also associated with obesity later in life[70], and a meta-analysis indicated it to be a risk factor for asthma[71]. Thus, smoking and overweight are potentially mediating factors in the relation between early life stress and asthma.

Psychological stress effects

Stress effects on physiology and behavior may directly worsen the level of lung function. However, there may be large variations in the resulting severity of dyspnea perceived by patients. Dyspnea perception in general does not directly relate to the level of objectively measured lung function[72]; processes such as attention to or interpretation of somatic symptoms may bridge this gap. Several studies show that exposure to stress might lead to biases in these processes and thus to substantial differences in dyspnea perception[73-75]. Stress and associated mood effects might trigger (or attenuate) a process of turning inward, thereby increasing attention towards the self, leading to a lower threshold for symptom perception. For example, anxious asthma patients tend to overperceive dyspnea[76]. In this context, one should keep in mind that physician diagnoses are based primarily on the presentation of symptoms to the physician.

THE TRACKING ADOLESCENTS' INDIVIDUAL LIVES SURVEY (TRAILS) STUDY

The association between stress and asthma will be studied in the TRacking Adolescents' Individual Lives Survey (TRAILS, see also www.trails.nl). TRAILS is a population-based cohort study including inhabitants of five communities in the North of the Netherlands, born between October 1st 1989 and September 30th 1990 (n=2,230). These adolescents are being studied biannually from age 10 at least until age 25, in order to assess the development of mental and somatic health from preadolescence into adulthood. Data covering the child's life before the age of 10 are collected from registries (e.g. youth health care), retrospective questionnaires and interviews. This includes data concerning pregnancy, birth weight, and growth and development during childhood. The key objective of TRAILS is to chart and explain the development of mental health from preadolescence into adulthood. As part of the enriching TRAILS program, blood, DNA and variables related to somatic health are collected. Detailed information has been described in Huisman et al. 2008[77].

TABLE 1.3 | Time schedule and variables under study

Survey	T1	T2	T3
Year	2001-2003	2004-2005	2006-2007
Determinants	Pubertal development (Tanner)	Pubertal development (Tanner and Pubertal Development Scale)	Pubertal development (Tanner and Pubertal Development Scale)
	Cortisol (basal)		Cortisol (basal and stress-induced)
	Perinatal stress (*in utero* exposure to maternal psychological problems)	Unpleasant events experienced between ages 0-5 and 6-11 years	Genetic data (*IL4*, *IL4R*, *IL13*, *CDH1*, *PCDH1*, *ADAM33*, *NR3C1*, *NR3C2*)
	Postnatal stress (postnatal depression)		*In utero* exposure to maternal smoking
	Stressful life events before age 4 years		
	Birth weight		
	Parental history of asthma		
Outcome	Asthma	Asthma	Asthma
			Asthma (European Community Respiratory Health Survey (ERCHS))
	Hay fever	Hay fever	Hay fever
	Allergy	Allergy	Allergy
	Eczema	Eczema	Eczema

AIMS AND OUTLINE OF THE THESIS

The aims of this thesis are to unravel the path from early life stress to the development of asthma up to adolescence, and to quantify the relative contribution of the mediating mechanisms via which stress leads to asthma development. However, before we study these aims, we first focus on the role of pubertal development in the gender-related switch in asthma prevalence, and on the genetic pathways involved in asthma development.

Chapter 2 describes the development of asthma up to adolescence. Specifically, the role of age and pubertal development in the gender-related switch in asthma prevalence will be studied. In addition, the association between pubertal development and asthma-related phenotypes, such as total IgE and peak expiratory flow fall during a shuttle run test, will be studied.

In **chapter 3**, early life risk factors for asthma development are studied. These include the combined effect of single nucleotide polymorphisms (SNPs) in genes involved in the development of asthma and asthma-related phenotypes (genetic risk score) on asthma development. The predicting properties of this genetic risk score will be compared to the predicting properties of know early life risk factors of asthma development such as *in utero* exposure to maternal smoking and maternal asthma.

In **chapter 4**, the role of exposure to perinatal stress and stress during early childhood on asthma development up to adolescence will be studied. In addition, *in utero* exposure to maternal smoking and birth weight will be studied as mediators in this association.

Chapter 5 and **6** concern the role of the HPA axis in asthma and asthma development. In **chapter 5**, the role of basal cortisol levels and cortisol levels under psychosocial stress in relation to the presence and development of asthma is studied. **Chapter 6** investigates whether SNPs in the mineralocorticoid or glucocorticoid receptor influence asthma risk.

The results of these chapters are summarized and discussed in **chapter 7,** which will conclude with some implications of this research for further studies towards the role of stress in the etiology of asthma.

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CHAPTER 2

Gender differences in asthma development and remission during transition through puberty: The Tracking Adolescents' Individual Lives Survey (TRAILS) study

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ABSTRACT

Background: During puberty, a gender shift in asthma prevalence occurs, with a preponderance of boys before puberty. The mechanisms underlying this gender shift are unclear. We assessed associations of pubertal stages and transition through puberty with (1) the prevalence, incidence, and remission of asthma in male and female subjects, (2) total IgE levels, and (3) peak expiratory flow (PEF) fall during a shuttle run test (SRT).

Methods: In the TRacking Adolescents' Individual Lives Survey study (n=2,230; 51% female subjects), associations between pubertal stages and the prevalence, incidence, and remission of asthma were tested by using logistic regression and generalized estimating equations at a mean ± SD age of 11.1 ± 0.6, 13.6 ± 0.5, and 16.3 ± 0.7 years. Multiple linear regression analysis were used to study log-transformed total IgE levels and PEF fall during a SRT dependent on early versus late pubertal stages at a mean age of 16.3 years.

Results: The prevalence of asthma was similar in boys (7.7%) and girls (7.4%) at a mean age of 11.1 years. The prevalence of asthma was significantly higher in female (6.2%) than male (4.3%) subjects at 16.3 years of age. There were no significant associations between transition of pubertal stages and the presence of asthma, either cross-sectionally or longitudinally. Pubertal stages and log-transformed total IgE levels or PEF fall during a SRT at age 16.3 years were not correlated.

Conclusions: A shift in the prevalence of asthma occurs between 11.1 and 16.3 years, which is due to both an increased incidence and decreased remission of asthma in female compared with male subjects. Pubertal stages could not be proved to explain the gender shift in asthma prevalence.

INTRODUCTION

Asthma is a chronic inflammatory disease of the airways. Before puberty, the prevalence of asthma is higher in boys than in girls. By adulthood, a gender switch in the prevalence of asthma has occurred, with female subjects having a higher prevalence than male subjects[1-5]. The exact age at which this switch occurs has not been identified, and a number of age ranges have been described with respect to the changing ratio of asthma[6]. Health care utilization related to asthma is significantly higher in male subjects aged 2 to 13 years, whereas it is greater in female subjects older than 23 years[6]. Additionally, boys with asthma are significantly more often hospitalized than girls until the age of 14 years, whereas the opposite is true in adulthood[7]. Because this change in male/female ratio occurs during puberty, hormonal changes throughout this period of life have been thought to be potential causative factors for the onset of asthma.

Female sex hormones have been linked to the development of asthma. In female subjects the risk of asthma is increased for those with early-onset menarche[5,8]. This effect might be due to higher exposure to cumulative female sex hormone concentrations in early-onset compared with late-onset menarche[5,9,10]. A longitudinal study showed that pregnancy, a state in which both progesterone and estrogen levels are increased, is also linked to the development of asthma[11]. However, cross-sectional studies did not show this association[10]. A role for female sex hormones is also suggested by the fact that after menopause the incidence of asthma is decreased in female subjects when compared with that seen in premenopausal women or men[12,13].

Female sex hormones have also been linked to asthma morbidity. Approximately 30% to 40% of female asthmatic subjects experience perimenstrual asthma worsening[10,14], which associates with increased symptoms[15] and a greater likelihood of hospitalization[14]. The exact mechanism behind this perimenstrual worsening is not clear, but it might involve the increase in progesterone and estrogen levels during the luteal phase, leading to increased inflammation of the airway wall[10,16]. Because oral contraceptives (OCs) suppress the increase in estrogen and progesterone levels during the luteal phase, the role of OCs on perimenstrual asthma has been studied. These studies suggest a small favorable effect of OCs on perimenstrual asthma and mild asthma[8,14]; however, in women with more severe asthma, symptoms are increased[10,17]. Studies assessing the role of pregnancy on asthma morbidity produced conflicting results[10]. In an equal number of women with mild asthma, asthma symptoms improved, remained stable, or deteriorated, and only in the more severe cases of asthma did asthma symptoms increase[12,18].

Animal studies suggest that male hormones play a role in asthma development as well. Male androgens might protect against the development of asthma[19]. Murine models of allergic airway disease show that male animals are less likely than female animals to have

airway inflammation[20]. Castrated mice have low blood levels of testosterone and develop more severe airway inflammation than sham-operated mice, and their airway inflammation is comparable with that seen in female mice[20]. It is possible that higher testosterone levels in sham-operated mice lead to systematic inhibition of the Th2 cell response and hence less severe airway inflammation[20]. Furthermore, administration of progesterone increases eosinophilic airway inflammation in male mice[21]. These animal models suggest that changing levels of male and female sex hormones influence the level of inflammation in both male and female mice.

Contrary to the apparent role of hormones shown in animal studies, the effect of changing levels of sex hormones on asthma is less clear cut in human subjects[5]. To our knowledge, only one study directly related the stage of puberty to the prognosis of asthma[3], but because this study was performed in adolescents with asthma, the association between pubertal stages and asthma incidence could not be studied.

As far as we know, no studies thus far have investigated the role of sex hormones on the development of asthma longitudinally. This is important because only with longitudinal methods can the association between hormonal changes during transition through puberty and the net change in the prevalence of asthma over time (net result of incidence and remission) be studied. We performed a prospective cohort study on the effect of changes in pubertal stages on asthma as a proxy for the hormonal changes that occurred during the ages of 11 to 16 years, the period in which transition through puberty takes place.

The aim of the current prospective study was to assess gender differences in the prevalence, incidence, and remission of asthma during transition through puberty. Additionally, we investigated gender differences in objective asthma-related phenotypes (total IgE levels and peak expiratory flow (PEF) fall after a shuttle run test (SRT)) in relation to the pubertal stage at a mean age of 16.3 years.

METHODS

Study population

The TRacking Adolescents' Individual Lives Survey (TRAILS) is a prospective cohort study among adolescents in the general Dutch population. Thus far, 3 surveys have been completed: survey 1 in 2001-2002 (mean ± SD age 11.1 ± 0.6 years), survey 2 in 2003-2004 (mean ± SD age 13.6 ± 0.5 years), and survey 3 in 2005-2007 (mean ± SD age 16.3 ± 0.7 years). Adolescents will be followed until at least the age of 24 years. Detailed information has been described elsewhere[20,23].

Questionnaire data

Data were collected on the presence of asthma, pubertal development (all 3 surveys), birth weight (first survey), cigarette smoking during pregnancy (third survey), and the use of OCs (third survey) (Table E2.5 in online supplement). Asthma was defined as a parentally reported physician's diagnosis of asthma and/or having symptoms of asthma and/or use of asthma treatment prescribed by a physician in the past 12 months. Incidence of asthma was defined as having asthma at a specific survey while not having asthma at the previous survey. Remission of asthma was defined as having asthma at a specific survey but not at the following survey.

Clinical characteristics

Height and weight were measured by using standardized protocols[24]. At each survey, body mass index (BMI) was calculated. Obesity is a risk factor for the development of asthma[25]. Therefore BMI was categorized as normal weight, overweight, and obesity based on age- and sex-specific cutoff points[26].

Total IgE

At the third survey (mean age, 16.3 years), 1,206 (66.4%) adolescents gave blood to determine serum total IgE levels.

Twenty-meter SRT

At the third survey, 629 (34.6%) adolescents completed a 20-m SRT, a reliable test to provoke exercise-induced asthma[27]. This test comprises running a 20-m track back and forth, starting at a speed of 8 km/h and increasing by 0.5 km/h every minute[28,29].

Heart rate monitoring was performed in a subsample of 241 (38.3%) adolescents who performed the SRT by using a Polar Team System type 1 as a control for maximal performance. The vast majority of the adolescents (>95%) performed a maximal test ($\geq$ 185 beats/min).

PEF measurements were performed before and after the SRT by using aMini-Wright PEF meter. The highest PEF of 3 measurements both before and after the SRT were used in the analysis. PEF fall was calculated as the difference between PEF after and before the SRT divided by PEF before the SRT.

Pubertal stages

Two measurements of pubertal development were used in this study: the Tanner stages of pubertal development[30,31], which were filled in by the parents at the first and second survey, and the Pubertal Development Scale (PDS) questionnaire[32,33], which was answered by the adolescents at the second and third surveys. The Tanner stages of pubertal development consist of schematic drawings of secondary sex characteristics (pubic hair and breast development for female subjects and pubic hair and genital development for male subjects).

A parent (usually the mother) selected the drawings that matched most closely to the actual pubertal development of the adolescent. Based on this rating, adolescents were classified into 5 Tanner stages of pubertal development, with stage 1 corresponding to the prepubertal stage and stage 5 corresponding to the postpubertal stage[24]. Adolescents answered in the PDS questions about their physical development, including changes in growth, body hair, and skin changes (acne). Also, female subjects were asked about breast development and menstruation, and male subjects were asked about voice changes and growth of facial hair[34].

The PDS questionnaire results were recoded into 5 stages of pubertal development by using the algorithm developed by Carskadon and Acebo[35]. By using this algorithm, female subjects without menarche and a total score of 5 or greater (body hair growth and breast development), male subjects with a total score of 4 to 5 and a 3-point response (body hair growth, voice changes and facial hair growth), and male subjects with a total score of 6 to 8 and a 4-point response (body hair growth, voice changes and facial hair growth) were not recoded into a stage of pubertal development. Therefore in line with other studies, in our study female subjects with no menarche and a total score of 5 or greater were classified as midpubertal[32], and male subjects with a total score of 4 to 5 and a 3-point response and a total score of 6 to 8 and a 4-point response were classified as early and midpubertal, respectively. In the current study *early pubertal* was defined as stage 1, 2, or 3, whereas *late pubertal* was defined as stage 4 or 5.

Statistical analysis

Differences in continuous variables between groups were tested with the unpaired *t* test or the Mann-Whitney *U* test, as appropriate. Differences in cell counts were tested with the χ^2 or Fisher exact tests. Changes in proportions between 2 surveys within groups were tested by using the McNemar test. Distributions were checked for normality by means of visual inspection of the probability plots of the residuals. Based on the literature, the following variables were entered into all models: BMI (normal weight, overweight, and obesity)[25], birth weight[36], and cigarette smoking during pregnancy[37]. Multiple logistic regression analysis was used to study the prevalence, incidence, and remission of asthma dependent on early versus late pubertal stage, as adjusted for potential confounders. Multiple linear regression analysis were used to study log-transformed total IgE levels and PEF fall during SRT dependent on early versus late pubertal stage, as adjusted for potential confounders. Both logistic and linear models were checked for multicollinearity. In none of the models was this a problem. Net changes in the prevalence of asthma over time (net result of incidence and remission) were estimated by using generalized estimating equations (GEE) models with age and age at first survey in the models. With GEE analysis, measurements of adolescents over time are analyzed, taking into account that these repeated measurements of 1 adolescent are not independent of each other. The relationship between pubertal stages and the development

of asthma was analyzed separately for both the cross-sectional (between subjects at age 11.1 years) and longitudinal (within a subject) associations. The regression coefficient of the cross-sectional analysis reflected the effect of pubertal stages on the prevalence of asthma at age 11.1 years. The regression coefficient of the longitudinal analysis consisted of pooled analysis of both the cross-sectional and longitudinal effects of pubertal stages on asthma development. For all other variables, a pooled coefficient was given, consisting of both the cross-sectional and longitudinal (within-subject) components[38]. The association between Tanner stages (early vs late pubertal) and the prevalence of asthma was studied in the first and second survey. The association between PDS stages (early vs late pubertal) and the presence of asthma was studied at the second and third survey. Multiple logistic regression analysis, multiple linear regression analysis, and GEE analysis were performed in the total dataset and stratified according to gender given the aims of our study: assessing gender differences in (risk factors for) incidence and remission of asthma during transition through puberty. Statistical analysis were performed with SPSS 16.0 software (SPSS, Inc, Chicago, Ill). P-values of less than 0.05 (2-sided) were considered significant.

RESULTS

Study population

A total of 2,230 adolescents with a mean ± SD age of 11.1 ± 0.6 years was included (51% female subjects, Table 2.1). At the second survey, 96% (n=2,149) were reassessed (mean ± SD age 13.6 ± 0.5 years; 51% female subjects). Eighty-one percent of the original number (n=1,816) completed the third survey (mean ± SD age 16.3 ± 0.7 years; 52% female subjects) (Figure E2.1 in online supplement). Adolescents who completed the third survey were significantly more often female and had a significantly lower BMI compared with the baseline population (Table E2.1 in online supplement).

Asthma prevalence

There was no significant difference in asthma prevalence between female and male subjects at 11.1, 13.6, or 16.3 years. In female subjects there was a significant difference in the prevalence of asthma between the ages of 11.1 and 13.6 years but not between the ages of 11.1 and 16.3 years and 13.6 and 16.3 years. In male subjects there was a significant difference in the prevalence of asthma between the ages of 11.1 and 16.3 years and between the ages of 13.6 and 16.3 years but not between the ages of 11.1 and 13.6 years (Figure 2.1). Twenty-one (43.8%) of 48 adolescents with asthma remission at 13.6 years were still in remission, 9 (18.8%) reported having asthma again at 16.3 years, and for 18 (37.5%), information about asthma status at age 16.3 years was missing.

TABLE 2.1 | Population characteristics of the study population (n=2,230)

Variable	Female subjects n = 1,132 (51%)	Male subjects n = 1,098 (49%)
Age (y)	11.1 ± 0.5	11.1 ± 0.6
Tanner stage 1	327 (30.0) *	351 (34.3)
Tanner stage 2	496 (45.5) *	619 (60.5)
Tanner stage 3	203 (18.6) *	49 (4.8)
Tanner stage 4	59 (5.4) *	2 (0.2)
Tanner stage 5	6 (0.5) *	2 (0.2)
Tanner stage	2 (1-5) *	2 (1-5)
Asthma	77 (7.4)	77 (7. 7)
BMI (kg/m²)	17.6 (12.7-33.9) *	17.1 (11.0-35.0)
Normal weight	896 (81.5)*	920 (86.6)
Overweight	166 (15.1)*	115 (10.8)
Obesity	37 (3.4)*	27 (2.6)
Birth weight (100g)	33.1 ± 6.1 *	34.7 ± 6.1
Cigarette smoking during pregnancy	267 (33.6)	212 (30.0)

Data are presented as number (percentage), mean ± SD or median (range). * $p<0.05$, significant difference between females and males.

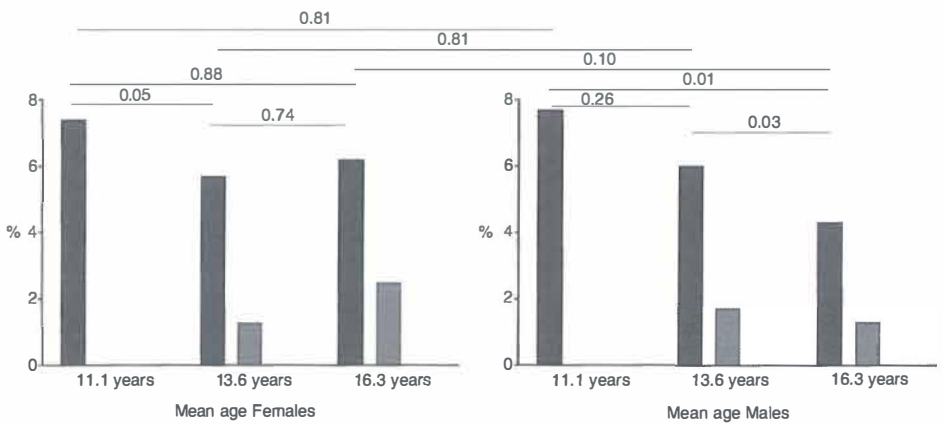

FIGURE 2.1 | Prevalence, incidence, and remission of asthma. Dark column = prevalence. Gray column = incidence. Light column = remission.

Pubertal stages

At 11.1 years, 65 (5.9%) female and 4 (0.4%) male subjects were classified as late pubertal according to the Tanner stages. At the age of 13.6 years, 437 (51.4%) female and 91 (14.5%) male subjects were classified as late pubertal according to the Tanner stages, as well as 659 (65.4%) female and 121 (12.0%) male subjects according to the PDS questionnaire. At the age of 16.3 years, 851 (98.0%) female and 506 (65.5%) male subjects were classified as late pubertal according to the PDS questionnaire. At all ages, female subjects had significantly higher stages of pubertal development than male subjects. Information on both the Tanner stages and the PDS questionnaire was available for 1,403 adolescents at the age of 13.6 years.

Adolescents and parents agreed on classification as early pubertal 52% (726) of the time and agreed 27% (373) of the time about classification as late pubertal. Thus in 79% of the cases, there was agreement between adolescents and parents with respect to pubertal stages. The measurement of agreement (κ) was 0.54 ($p<0.001$).

Asthma and pubertal stages

Complete data on gender, age, asthma, Tanner stage, BMI, birth weight, and cigarette smoking during pregnancy were available for 719 (63.5%) female and 632 (57.6%) male subjects at the age of 11.1 years and for 637 (56.3%) female and 444 (40.4%) male subjects at the age of 13.6 years. Complete data on gender, age, asthma, pubertal development stage, BMI, birth weight, and cigarette smoking during pregnancy were available for 679 (60.0%) female and 636 (57.9%) male subjects at the age of 13.6 years and for 669 (59.1%) female and 586 (53.4%) male subjects at the age of 16.3 years. At the age of 11.1, 13.6, and 16.3 years, we observed no significant association between pubertal stages and asthma prevalence cross-sectionally in the total population or in female and male subjects separately. At the age of 13.6 and 16.3 years, we observed no significant association between pubertal stages and asthma incidence and remission cross-sectionally in the total population or in female and male subjects separately. A sensitivity analysis at the age of 13.6 years showed similar associations between parent-reported (Tanner) and adolescent-reported (PDS) pubertal stages and asthma. Restricting the analysis to the 1,099 adolescents for whom both the parents and the adolescent agreed on pubertal stages did not affect the effect estimates. In longitudinal analysis we found no significant association between the pubertal stage and asthma prevalence in female or male subjects (Table 2.2). A sensitivity analysis in which male subjects with a total score of 4 to 5 and a 3-point response and male subjects with a total score of 6 to 8 and a 4-point response were recoded into midpubertal and late pubertal classifications, respectively, did not affect the

TABLE 2.2 | Independent longitudinal effect estimates of risk factors on the presence of asthma

Variable	Pubertal stage							
	Age 11.1 till 13.6 y*				Age 13.6 till 16.3 y†			
	Female sex		Male sex		Female sex		Male sex	
	OR (95% CI)	p-value	OR (95% CI)	p-value	OR (95% CI)	p-value	OR (95% CI)	p-value
Age upon inclusion (y)‡	1.38 (0.81-2.35)	0.24	0.87 (0.48-1.57)	0.64	1.14 (0.68-1.94)	0.62	0.92 (0.51-1.67)	0.79
Longitudinal effect of time	1.00 (0.88-1.13)	0.95	0.98 (0.85-1.12)	0.74	0.98 (0.85-1.13)	0.75	0.81 (0.68-0.97)	0.02
Pubertal stages§	0.75 (0.44-1.27)	0.28	0.76 (0.21-2.78)	0.68	1.00 (0.55-1.81)	0.99	1.42 (0.80-2.52)	0.23
Obesity	3.25 (1.10-9.58)	0.03	0.56 (0.18-1.78)	0.33	4.01 (1.30-12.35)	0.02	1.12 (0.15-8.66)	0.91
Overweight	2.01 (1.17-3.44)	0.01	1.59 (0.83-3.04)	0.17	2.44 (1.39-4.29)	<0.01	2.21 (1.20-4.07)	0.01
Birth weight (100g)	1.00 (0.96-1.04)	0.98	0.99 (0.94-1.04)	0.68	1.00 (0.96-1.04)	0.85	1.00 (0.94-1.06)	0.90
Cigarette Smoking during pregnancy	2.04 (1.15-3.60)	0.01	1.30 (0.68-2.49)	0.43	1.70 (0.97-2.96)	0.06	1.18 (0.58-2.40)	0.66

* Tanner stages. † PDS stages. ‡ Reflects difference between longitudinal and cross-sectional effect of pubertal stages. § Defined as early versus late pubertal.

effect estimates. Excluding age from the model did not affect the effect estimates. Excluding adolescents for whom BMI data were not available at all 3 surveys did not affect the effect estimates.

Thirty-one percent (n=248) of the female subjects used OCs at the age of 16.3 years. There was no significant difference in BMI categories (normal weight, overweight, and obesity) between female subjects using OCs and those who did not use OCs. Controlling for OCs use did not change the association between pubertal stages and asthma.

Total IgE

The total median IgE level was 73.0 kU/L (25th-75th percentile, 24.6-224.0 kU/L). In the total population there was a near significant difference in total IgE levels between female and male subjects (p=0.052). Adolescents with asthma had significantly higher total IgE levels than adolescents without asthma. We did not find a significant association between pubertal stage and log-transformed total IgE levels in female or male subjects (Table E2.2 in online supplement). A sensitivity analysis in which male subjects with a total score of 4 to 5 and a 3-point response and male subjects with a total score of 6 to 8 and a 4-point response were recoded into midpubertal and late pubertal classifications, respectively, did not affect the effect estimates. Excluding age from the model did not affect the effect estimates.

PEF fall during SRT

The median PEF before the SRT was 540 L/min (range (280-750 L/min)) in male subjects and 440 L/min (170-610 L/min) in female subjects. The median PEF after the SRT was 550 L/min (300-750 L/min) in male subjects and 450 L/min (190-630 L/min) in female subjects. Male subjects had significantly higher PEF values before and after the SRT (Table E2.3 in online supplement). Twenty-five percent of the population (total, 155; 88 female and 67 male subjects) had a fall in median PEF during the SRT of 20.0 L/min (10-140 L/min). PEF fall during the SRT was 20 L/min (10-140 L/min) in male subjects and 20 L/min (10-130 L/min) in female subjects. There was no significant difference in PEF fall during the SRT between female and male subjects (Table E2.3 in online supplement). Of the 155 adolescents with a PEF fall during the SRT, 11 (7%) adolescents had asthma, 132 (85%) did not have asthma, and 12 (8%) lacked information about asthma status at a mean age of 16.3 years. There was no significant difference in PEF fall during the SRT between adolescents with and without asthma. We found no significant association between PEF fall during the SRT with pubertal maturity in female or male subjects (Table E2.4 in online supplement). A sensitivity analysis in which male subjects with a total score of 4 to 5 and a 3-point response and male subjects with a total score of 6 to 8 and a 4-point response were recoded into midpubertal and late pubertal classifications, respectively, did not affect the effect estimates. Excluding age from the model did not affect the effect estimates.

DISCUSSION

Our longitudinal study in a large group of boys and girls followed through puberty did not show differences in asthma prevalence between boys and girls at the age of 11.1 and 13.6 years, whereas at 16.3 years of age, more female than male subjects had asthma. This higher prevalence of asthma at the age of 16.3 years was related to a higher incidence of asthma and lower remission of asthma in female compared with male subjects during this follow-up. This study did not show an association between pubertal stages and asthma prevalence. Additionally, we found no significant association between pubertal stages and asthma-related phenotypes (log-transformed total IgE levels and PEF fall during SRTs).

The strength of the current study is the longitudinal study design, which allowed us to study the association between pubertal stages and changes in asthma prevalence during transition through puberty. Adolescents were followed up from the age of 11.1 years until 16.3 years. This is important because the mean age at which girls enter puberty is 10.5 years, completing sexual development after 4.2 years on average, whereas boys enter puberty at 11.5 years and achieve complete sexual development after 3.5 years on average[3].

Our results are largely in agreement with findings from a previous study showing that in early childhood the prevalence of asthma is higher in boys than in girls[10]. Between the ages of 9 and 11 years, the difference in the prevalence of asthma between boys and girls disappeared[39]. In agreement with these results, we found no difference in the prevalence of asthma between boys and girls at a mean age of 11.1 years. Nicolai et al[3] followed-up adolescents with asthma from the age of 10 years until 14 years. At 20 years of age, these adolescents were re-examined, together with a sample of nonasthmatic adolescents from the original cross-sectional study. At this time point, there were still more male subjects with asthma than female subjects in the initially studied group. However, in the group without asthma at the age of 10 years, twice as many female subjects had asthma at age 20 years[4], indicating a higher incidence of asthma in female compared with male subjects between the ages of 10 and 20 years. This is in agreement with our data, in which the incidence of asthma was higher and remission was lower in female compared with male subjects between the ages of 13.6 and 16.3 years.

We are the first to study the association between pubertal stages and the net change in the prevalence of asthma over time (net result of incidence and remission) using longitudinal methods. Despite the fact that we identified the hypothesized gender switch in the prevalence of asthma, we observed no association between pubertal stages and asthma prevalence. In addition, we found no significant associations between pubertal stages and asthma-related phenotypes, such as log-transformed total IgE levels or PEF fall during SRTs at the age of 16.3 years. As far as we know, only 1 previous study has directly related pubertal

stages to asthma, but this study of Nicolai et al[3] included asthmatic subjects only and their prognosis. In this study menarche, voice changes, and serum androstanediol glucuronide levels (an endocrine marker that reflects peripheral testosterone metabolism) were used as a surrogate for late puberty. In accordance with the results of our study, Nicolai et al[3] found no statistically significant relationship between the reported signs of late puberty and remission of asthma in adolescents who had had active asthma at age 10 years. Likewise, no association was found between androstanediol glucuronide levels and asthma[3].

There is a randomized, double-blind, multicenter clinical trial that investigated the relationship between airway responsiveness and pubertal stages[40]. This study consisted of a treatment phase (budesonide, 400 µg daily, or nedocromil sodium, 16 mg daily) and an observation phase (with asthma care based on national guidelines). Children aged 5 to 12 years with mild-to-moderate asthma were followed up 8.6 ± 1.8 years, and methacholine provocation tests were performed before randomization, 8 months after randomization, and then yearly. They found that between the ages of 5 and 11 years, PC_{20} values increased in both girls and boys. However, after the age of 11 years, PC_{20} values continued to increase in boys compared with the minimal increase seen in girls. This increase in PC_{20} values in boys relative to girls started at Tanner stage 2. The mean age at which children enter Tanner stage 2 is 11 years. The results of this study are not consistent with our results, namely no association between pubertal stages and asthma. Possible explanations for this inconsistent finding could be the fact that our study was a population-based study, and in the study of Tantisira et al[40], children with mild-to-moderate asthma were included. Second, Tantisira et al studied airway responsiveness, whereas we studies exercise-induced asthma. Third, in the study of Tantisira et al, assessment of pubertal stages was done by a physician. We used both parent-reported and adolescent-reported pubertal development.

The suggestion that hormonal changes, as occur during puberty, play a role in asthma development and remission[5,8-10,14,15,19,20] was not confirmed by our study. We used questionnaires as proxy measures of what is really happening during puberty (ie, changes in sex hormones). However, questionnaires might be too insensitive to reflect hormonal changes.

If the gender switch in the prevalence of asthma is not due to changing levels of sex hormones, the question arises of which other mechanisms could potentially explain this development. One potential mechanism is the difference in physical growth of the lungs from birth into adulthood between boys and girls. At birth, boys have narrower airways in proportion to total lung volume than girls. The development of the lungs continues until adolescence[41], when a difference in maturation of the airways in comparison with lung parenchyma and airspace exists between female and male subjects[10]. This leads to a smaller airway diameter in relation to lung volume in female compared with male subjects

at the age of 16 years[10,16]. This gender difference in lung growth could explain why male subjects are at risk for asthma early in life but female subjects are at risk especially after puberty. However, there is also evidence that a smaller airway size does not simply lead to a higher risk of asthma because, especially in children, there is not much difference in the absolute size of the airways between boys and girls[10].

A second potential mechanism to explain the gender switch in the prevalence of asthma occurring during puberty is obesity, a recognized risk factor for the development of asthma[25]. Obesity is accompanied by an increased production of estrogens and is therefore related to the development of puberty in a gender specific way. Obesity is associated with early menarche in girls and a delayed puberty in boys[42]. Obese girls with early menarche (before the age of 11 years) have a higher risk of asthma than those with late-onset menarche[42,43]. Both in cross-sectional and longitudinal analysis, we identified obesity as a risk factor for asthma in female subjects. For male subjects, we found no association between obesity and asthma both in cross-sectional and longitudinal analysis. These findings could indicate a positive role for BMI in the development of asthma in female subjects. However, the difference in BMI development during transition through puberty could not explain the observed sex-related switch in asthma prevalence because of the fact that boys and girls had the same increase in BMI during transition through puberty.

Our study has some limitations. In the current study puberty was assessed by using 2 different questionnaires, one filled in by the parents at the first (mean age, 11.1 years) and second (mean age, 13.6 years) surveys (Tanner), and the other (PDS) filled in by the adolescents at the second (mean age, 13.6 years) and third (mean age, 16.3 years) surveys. The fact that we analyzed the data for the Tanner stages and PDS questionnaire separately decreased the power to detect a significant association between pubertal stages and asthma prevalence and resulted in large CIs for the estimated odds ratios.

The second limitation was that asthma was defined as having a parentally reported physician's diagnosis of asthma and/or having symptoms of asthma and/or use of asthma treatment prescribed by a physician in the past 12 months. Based on our data, we cannot determine whether children had just exercise-induced asthma in isolation or transient wheeze related to a respiratory tract infection or more established asthma. We know from (unpublished) data of the Dutch part of the European Community Respiratory Health Survey that in the general population 98% of the subjects who report asthma indeed have a physician's diagnosis of asthma. Therefore we believe that using questionnaire data to define asthma status is a reliable way to determine the presence of asthma in a Dutch general population–based cohort like TRAILS.

One advantage of the longitudinal study design used in this study is the fact that both the cross-sectional and longitudinal effects of pubertal stages on the development of

asthma could be analyzed. However, at the age of 11.1 years, 99.6% (1,019) of the boys were classified as early pubertal, and therefore analyzing both the longitudinal and cross-sectional effects of the Tanner stages on the development of asthma was not possible. At the age of 13.6 years, 12.0% (121) of the male subjects were classified as late pubertal. At this age, we could study both the cross-sectional and longitudinal effects of the PDS stages on the development of asthma between the ages of 13.6 and 16.3 years. We found no significant association between the cross-sectionally and longitudinally analyzed PDS stages and asthma development (data not shown).

In summary, our longitudinal study shows that a shift in asthma prevalence has already started at the age of 11.1 years and goes on until 16.3 years. This shift is due to both a higher incidence of asthma and a lower remission of asthma in female compared with male subjects during this pubertal period. This shift cannot simply be explained by changes in pubertal stages. At the age of 16.3 years, no association was found between pubertal stages and asthma-related phenotypes (log-transformed total IgE levels and PEF fall during SRTs). We found no association between the sex switch in asthma and pubertal stage, and therefore questionnaires (Tanner and PDS) to assess pubertal stage might be too insensitive to estimate hormonal changes. More studies, using even smaller time intervals and measuring actual levels of sex hormones, are needed to disentangle the role of sex hormones in the development and remission of asthma during puberty.

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ONLINE SUPPLEMENT

TABLE E2.1 | Comparison of baseline characteristics between adolescents who completed the third survey and those who were lost to follow-up

	Follow-up n = 1,816	Lost to follow-up n = 414
Female sex	950 (52.3)*	182 (44.0)
Male sex	866 (47.7)*	232 (56.0)
Age (y)	11.1 ± 0.6	11.1 ± 0.5
Pubertal stages	2 (1-5)	2 (1-5)
Asthma	124 (7.3)	30 (8.6)
BMI‡§	17.3 (11.0-35.0)*	17.7 (12.8-31.6)
Birth weight (100 g)	33.9 ± 6.1	33.8 ± 6.3
Cigarette smoking during pregnancy	479 (31.9)	NA

Data are presented as number (percentage), mean ± SD or median (range). NA = Not assessed. *p<0.05, significant difference between follow-up and lost to follow-up. §Defined as early versus late pubertal.

TABLE E2.2 | Independent cross-sectional associations of risk factors for serum log transformation of total IgE levels in female and male subjects at the age of 16.3 years

	Female subjects		Male subjects	
	b (95% CI)	p-value	b (95% CI)	p-value
Age (y)	-0.04 (-0.13-0.06)	0.34	0.01 (-0.09-0.11)	0. 86
Pubertal stage*	-0.20 (-0.59-0.20)	0.33	0.05 (-0.08-0.18)	0.44
Obesity	0.29 (-0.09-0.67)	0.14	0.28 (-0.09-0.65)	0.13
Overweight	0.06 (-0.13-0.24)	0.52	-0.01 (-0.22-0.20)	0.94
Birth weight (100 g)	0.01 (-0.00-0.02)	0.09	0.00 (-0.01-0.01)	0.63
Cigarette smoking during pregnancy	0.04 (-0.09-0.17)	0.54	-0.18 (-0.32- -0.05)	0.01

b = regression coefficient. *Defined as early versus late pubertal.

TABLE E2.3 | SRT

Variable	Female subjects	Male subjects	p-value
PEF before SRT (L/min)	440 (170-610)*	540 (280-750)	<0.001
PEF after SRT (L/min)	450 (190-630)*	550 (300-750)	<0.001
PEF fall during SRT (%)	20 (10-130)	20 (10-140)	0.99

*Values are expressed as median (range).

TABLE E2.4 | Independent cross-sectional associations of risk factors for percentage fall in PEF after SRT in female and male subjects at the age of 16.3 years

	Female subjects		Male subjects	
	b (95% CI)	p-value	b (95% CI)	p-value
Age (y)	-0.48 (-2.53-1.57)	0.65	0.61 (-1.25-2.46)	0.52
Pubertal stage*	1.86 (-4.04-7.76)	0.54	1.70 (-0.34-3.73)	0.10
Obesity	2.36 (-6.40-11.11)	0.60	-7.88 (-15.22- -0.53)	0.04
Overweight	0.43 (-3.47-4.33)	0.83	-3.42 (-6.85-0.01)	0.05
Birth weight (100 g)	-0.23 (-0.41- -0.06)	0.01	-0.19 (-0.35- -0.03)	0.02
Cigarette smoking during pregnancy	0.44 (-1.91-2.78)	0.71	0.54 (-1.66-2.74)	0.63

b = regression coefficient. *Defined as early versus late pubertal.

TABLE E2.5 | Questions on questionnaires

Questionnaire filled in by:	Question
Parent	Did your child have asthma symptoms during the last 12 months?
Parent	Did your child use asthma medication during the last 12 months?
Parent	Was your child treated for asthma by a physician during the last 12 months?
Parent	Did a physician give your child a diagnosis of asthma?
Parent*	Did the mother smoke any cigarettes during the first trimester of pregnancy?
Parent*	Did the mother smoke any cigarettes during the second trimester of pregnancy?
Parent*	Did the mother smoke any cigarettes during the third trimester of pregnancy?
Parent	What was your child's birth weight?

*Cigarette smoking during pregnancy.

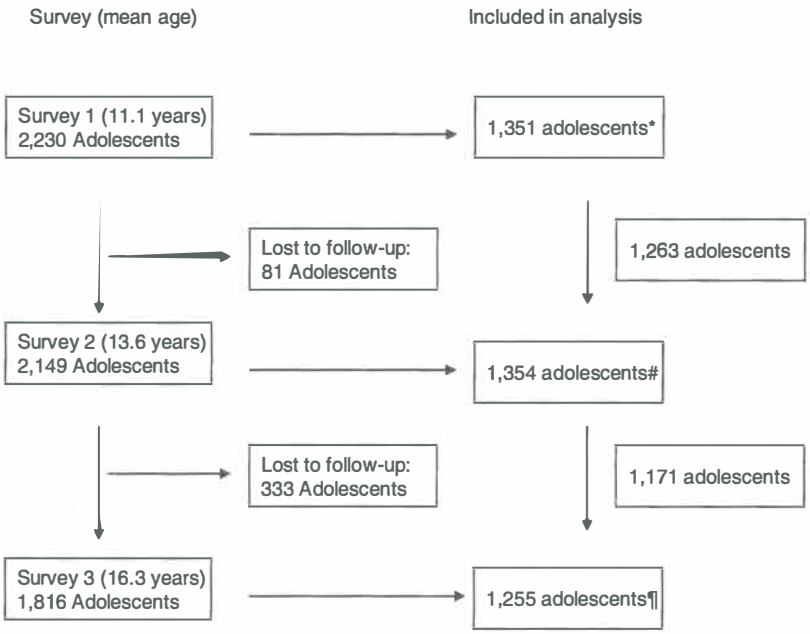

FIGURE E2.1 | Overview of adolescents included in the study.
*Tanner questionnaire. #Tanner or pubertal development questionnaire. ¶ Pubertal development questionnaire.

CHAPTER 3

A genetic risk score predicting asthma development in a cohort of adolescents

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Submitted.

ABSTRACT

Background: Genes involved in allergy (*IL4*, *IL4R* and *IL13*), epithelial function (*CDH1* and *PCDH1*) and airway remodeling (*ADAM33*) are associated with asthma. Combining these genes with modest effects on asthma into a genetic risk score (GRS) might result in a better prediction of asthma development. We studied (1) the predictive properties of the GRS for asthma development, and (2) the interaction between GRS and common risk factors.

Methods: We studied adolescents from the TRacking Adolescents' Individual Lives Survey (TRAILS) study (n=2,230, age surveys 1 =11 years, 2=14 years, 3=16 years) using logistic regression models adjusted for sex. GRS was calculated by summing the number of risk-raising alleles.

Results: GRS was significantly associated with asthma (OR (95% CI) 1.19 (1.03-1.36)), as were common risk factors such as *in utero* exposure to maternal smoking (1.64 (1.15-2.33)) and maternal asthma (4.61 (2.95-7.19)). Moreover, GRS remained significantly associated with asthma (1.25 (1.06-1.47)) after adjustment for these common risk factors. There was no significant interaction between GRS and the common risk factors.

Conclusion: The GRS is a highly significant independent predictor of asthma. Its merits should be further explored on its potential in clinical and screening practice.

INTRODUCTION

Asthma is a complex disease, in which multiple genes in biological pathways are involved, for instance genes in pathways of T helper 2 (Th2) cell differentiation and allergic inflammation like interleukin 4 (*IL4*), *IL4* receptor (*IL4R*) and interleukin 13 (*IL13*)), epithelial function (E-cadherin (*CDH1*) and Protocadherin-1 (*PCDH1*)) and airway remodeling (A Disintegrin And Metalloprotease 33 (*ADAM33*)). Several single nucleotide polymorphisms (SNPs) in these genes have been linked to the susceptibility for development of asthma and asthma-related phenotypes[1-11]. However, the effects of these individual SNPs on asthma or asthma-related phenotypes are modest. Therefore, combining the effect of SNPs in these multiple genes into a genetic risk score (GRS) might result in a better predictor of asthma development.

Other risk factors that are known to be associated with an increased risk for asthma development are *in utero* exposure to maternal smoking and maternal asthma[12,13]. Moreover, recent studies showed an interaction between *in utero* exposure to passive smoking and *IL13*, *PCDH1* and *ADAM33* resulting in an increased risk of early onset persistent wheeze[14], lung function decline[15] and in the development of bronchial hyperresponsiveness (BHR)[8,16], all asthma-related phenotypes. However, it is yet unknown whether these gene-environment interactions also play a role in asthma development in adolescence. In addition, the effect of the interaction between maternal asthma and genetic predisposition on asthma is also largely unknown.

We constructed a GRS based on SNP in genes that were reported to be significantly associated with asthma and asthma-related phenotypes in the literature, i.e. *IL4*, *IL4R*, *IL13*, *CDH1*, *PCDH1* and *ADAM33*. We tested the predictive properties of this GRS for asthma development in the TRAILS cohort of adolescents. We compared this predictive value of the GRS with common risk factors for asthma development, i.e. *in utero* smoke exposure to maternal smoking and maternal asthma. Additionally, we studied whether there were interactions of the GRS with these common risk factors on asthma development.

METHODS

Study population and design

We studied subjects from the TRacking Adolescents' Individual Lives Survey (TRAILS). TRAILS is a prospective cohort study among Dutch adolescents. The TRAILS cohort is a representative sample of adolescents of the general population. So far, three surveys have been completed: survey 1 in 2001-2002 (n=2,230, mean ± SD age 11 ± 0.6 years), survey 2 in 2003-2004 (n=2,149, mean ± SD age 14 ± 0.5 years) and survey 3 in 2005-

2007 (n=1,816, mean ± SD age 16 ± 0.7 years). Adolescents will be followed until at least the age of 24 years. Detailed information about the sample selection and study design has been described elsewhere[17].

Asthma

Information about parentally reported asthma (doctor diagnosis of asthma, symptoms of asthma and asthma treatment prescribed by a physician in the past 12 months) was collected at age mean age 11, 14 and 16 years of the adolescents using questionnaires[18]. Asthma was defined as parentally reported doctor diagnosis of asthma, and/or symptoms of asthma and/or asthma treatment prescribed by a physician in the past 12 months at age mean age 11, 14 and/or 16 years[18].

Early environmental risk factors for asthma development

Information on parentally reported *in utero* exposure to maternal smoking was collected at mean age 16 years using a standardized questionnaire[18]. Information about parentally reported maternal asthma was reported at age mean age 11 years.

Selection of SNPs

Genes implicated in Th2 cell differentiation and allergic inflammation (*IL4*, *IL4R* and *IL13*), epithelial function (*CDH1* and *PCDH1*) and airway remodeling (*ADAM33*) that were previously found to be significantly associated with asthma or asthma-related phenotypes[1-11] were used to construct the GRS. (t)SNPs in these genes were selected using Haploview. A total of 10 SNPs were included into the GRS calculation. (See online supplement for detailed information about the selected SNPs for *IL4*, *IL4R*, *IL13*, *CDH1*, *PCDH1* and *ADAM33*).

Genotyping

At mean age 16 years (n=1,816), DNA was extracted from blood samples (n=1,207) or buccal swabs (Cytobrush®, n=258) using a manual salting out procedure as described by Miller and colleagues[19]. SNP typing was performed on the Golden Gate Illumina BeadStation 500 platform (Illumina, Inc., San Diego, CA, USA) following the manufacturer's protocol. Genotype clustering was performed in BeadStudio 3.0. (Illumina, Inc., San Diego, CA, USA). SNPs were genotyped in 1,454 adolescents. See online supplement for detailed information about genotyping and quality control.

Statistical analysis

To study the predictive properties of the combined effect of SNPs in *IL4*, *IL4R*, *IL13*, *CDH1*, *PCDH1* and *ADAM33* on asthma, a GRS was calculated. Firstly, the individual SNPs in *IL4*, *IL4R*, *IL13*, *CDH1*, *PCDH1* and *ADAM33* were analyzed in an additive genetic model for the effect on asthma using logistic regression models adjusted for sex.

Secondly, SNPs that were associated with asthma within the TRAILS study ($p \leq 0.20$; rs762534 (*IL4*), rs3024578, rs3024647, rs12102586 (*IL4R*), rs3091307, rs2243297 (*IL13*), rs12447341, rs13689, rs8061932 (*CDH1*), rs14359, rs3822357, rs11167761, rs17097812 (*PCDH1*), rs3918396 (*ADAM33*), Table 3.2) were included in the GRS. The risk-raising allele was defined as the allele that is associated with a higher risk of asthma (homozygote non-risk genotype = 0, heterozygote and homozygote risk genotype = 1). Since for rs762534 (*IL4*), rs3024578, rs12102586 (*IL4R*) and rs2243297 (*IL13*) only the heterozygote and not the homozygote genotypes were associated with a higher risk of asthma, these SNPs were not included into the calculation of the GRS. GRS was calculated by summing the number of risk-raising alleles. A logistic regression model was used to study the association between GRS and asthma development adjusted for sex. Next, logistic regression models were used to study the associations between early environmental risk factors (e.g. *in utero* exposure to maternal smoking and maternal asthma) and asthma development adjusted for sex. Furthermore, logistic regression model was used to study the association between GRS and asthma development after adjustment for sex and the common risk factors *in utero* exposure to maternal smoking and maternal asthma.

Thirdly, we tested whether there was interaction between the GRS and *in utero* exposure to maternal smoking on the development of asthma by performing logistic regression analysis with GRS, *in utero* exposure to maternal smoking and the interaction term GRS x *in utero* exposure to maternal smoking into the model, adjusted for sex. Using a similar model, we tested whether there was interaction between the GRS and maternal asthma.

Statistical analysis were performed using SPSS Inc. Windows 18.0. P-values<0.05 (tested 2-sided) were considered to be significant.

RESULTS

Characteristics of the study population

Table 3.1 shows the characteristics of the study population. The SNPs selected form *IL4, IL4R, IL13, CDH1, PCDH1, ADAM33* and their association with asthma development are shown in Table 3.2. There were no differences between adolescents whose DNA was or was not available for genotyping with regard to asthma development, *in utero* exposure to maternal smoking or maternal asthma. However, adolescents whose DNA was available for genotyping were more often females compared to adolescents whose DNA was not available for genotyping.

TABLE 3.1 | Characteristics of the study population

Variable	Total population n = 2,230
Females	1,132 (51)
Ever Asthma	209 (10)
Asthma at mean age 11 years	154 (8)
Asthma at mean age 14 years	112 (6)
Asthma at mean age 16 years	79 (5)
In utero exposure to smoking	479 (32)
First trimester	462 (31)
Second trimester	407 (27)
Third trimester	402 (27)
Maternal asthma[1]	109 (6)

Data are presented as number (percentage of non-missing values). [1] At baseline (mean age 11 yrs).

Association of the predictive properties of GRS, *in utero* exposure to maternal smoking and maternal asthma with asthma development

Table 3.3 shows the estimated effects of GRS, *in utero* exposure to maternal smoking and maternal asthma on asthma development adjusted for sex. The GRS, *in utero* exposure to maternal smoking and maternal asthma were all significantly and positively associated with asthma development. After adjustment for *in utero* exposure to maternal smoking and maternal asthma, the GRS was still significantly and positively associated with asthma development.

Interaction of the GRS with *in utero* exposure to maternal smoking and maternal asthma

Table 3.4 shows the estimated interaction effect between GRS and the common risk factors on asthma development. There was no significant interaction between the GRS and *in utero* exposure to maternal smoking. There was also no significant interaction between the GRS and maternal asthma.

TABLE 3.2 | Estimated associations between single nucleotide polymorphisms (SNPs) in *IL4, IL4R, IL13, CDH1, PCDH1, ADAM33* and asthma in the TRAILs cohort

rs number	Gene	Chromosome	Major > Minor allele	MAF	Frequency	OR (95% CI)[1]	p-value
rs2243266	*IL4*	5	G/G	27	1,454	1.16 (0.83-1.63)	0.38
rs2227282	*IL4*	5	C/C	8	1,453	1.08 (0.82-1.42)	0.60
rs2243248	*IL4*	5	A/C	9	1,454	0.96 (0.60-1.52)	0.83
rs762534	*IL4*	5	C/A	12	1,454	0.65 (0.39-1.07)*	0.09
rs2243263	*IL4*	5	C/G	27	1,454	0.82 (0.55-1.23)	0.34
rs3024578	*IL4R*	16	G/A	8	1,454	0.71 (0.42-1.18)*	0.18
rs3024544	*IL4R*	16	G/A	15	1,454	0.85 (0.60-1.22)	0.39
rs3024530	*IL4R*	16	A/G	47	1,452	0.92 (0.72-1.18)	0.51
rs6498011	*IL4R*	16	G/A	40	1,454	1.04 (0.81-1.33)	0.78
rs3024647	*IL4R*	16	A/G	15	1,454	0.74 (0.50-1.08)*	0.11
rs3024676	*IL4R*	16	C/A	17	1,454	1.06 (0.76-1.48)	0.74
rs1029489	*IL4R*	16	G/A	44	1,454	0.94 (0.74-1.20)	0.63
rs8832	*IL4R*	16	G/A	50	1,453	1.02 (0.80-0.30)	0.89
rs2239347	*IL4R*	16	A/C	47	1,453	0.88 (0.68-1.12)	0.30
rs4787956	*IL4R*	16	A/G	40	1,454	1.01 (0.78-1.29)	0.97
rs8057585	*IL4R*	16	G/C	42	1,454	0.90 (0.70-1.16)	0.43
rs4787948	*IL4R*	16	A/G	31	1,454	1.04 (0.81-1.35)	0.75
rs3024622	*IL4R*	16	G/C	37	1,453	0.99 (0.76-1.29)	0.95
rs12102586	*IL4R*	16	G/A	9	1,454	0.70 (0.43-1.14)*	0.15
rs3024613	*IL4R*	16	G/A	47	1,346	1.15 (0.90-1.47)	0.25
rs3024585	*IL4R*	16	G/A	49	1,430	0.96 (0.74-1.24)	0.73

TABLE 3.2 | Continued

rs number	Gene	Chromosome	Major > Minor allele	MAF	Frequency	OR (95% CI)[1]	p-value
rs2057768	*IL4R*	16	G/A	29	1,454	0.98 (0.75-1.29)	0.89
rs3024560	*IL4R*	16	A/C	37	1,452	1.01 (0.78-1.32)	0.92
rs1295685	*IL13*	5	G/A	21	1,454	1.00 (0.74-1.35)	1.00
rs2243208	*IL13*	5	A/G	10	1,454	0.83 (0.54-1.27)	0.38
rs3091307	*IL13*	5	A/G	21	1,453	0.77 (0.56-1.06)*	0.11
rs1295683	*IL13*	5	G/A	11	1,452	1.21 (0.84-1.74)	0.31
rs2243297	*IL13*	5	A/T	4	1,443	0.55 (0.24-1.26)*	0.15
rs9929218	*CDH1*	16	G/A	22	1,454	0.99 (0.75-1.30)	0.94
rs11640099	*CDH1*	16	A/C	13	1,454	1.07 (0.76-1.52)	0.69
rs12447341	*CDH1*	16	G/A	35	1,454	**0.75 (0.57-0.98)***	0.03
rs13689	*CDH1*	16	A/G	22	1,454	1.26 (0.95-1.66)*	0.11
rs8061932	*CDH1*	16	A/G	16	1,454	1.23 (0.91-1.68)*	0.18
rs10431924	*CDH1*	16	G/A	41	1,439	1.11 (0.87-1.42)	0.41
rs9927789	*CDH1*	16	A/C	11	1,451	1.06 (0.72-1.58)	0.76
rs2276329	*CDH1*	16	A/G	9	1,451	1.11 (0.73-1.69)	0.62
rs3931740	*CDH1*	16	C/A	9	1,453	0.97 (0.63-1.49)	0.88
rs7188750	*CDH1*	16	G/A	18	1,453	1.19 (0.88-1.61)	0.26
rs47853573	*CDH1*	16	A/G	34	1,450	0.89 (0.69-1.16)	0.38
rs2276330	*CDH1*	16	A/G	15	1,451	1.23 (0.88-1.72)	0.22
rs11075699	*CDH1*	16	A/G	45	1,451	0.93 (0.72-1.19)	0.55

rs7186053	*CDH1*	16	G/A	41	1,451	1.12 (0.88-1.44)	0.36
rs16958383	*CDH1*	16	G/A	16	1,454	1.19 (0.87-1.62)	0.28
rs6888135	*PCDH1*	5	A/C	50	1,452	0.96 (0.75-1.23)	0.73
rs14359	*PCDH1*	5	G/C	23	1,453	1.27 (0.96-1.66)*	0.09
rs4382196	*PCDH1*	5	C/G	46	1,453	0.93 (0.72-1.19)	0.56
rs3822357	*PCDH1*	5	G/A	8	1,452	0.58 (0.33-1.00)*	0.051
rs10054186	*PCDH1*	5	G/C	18	1,454	0.89 (0.64-1.23)	0.48
rs10063472	*PCDH1*	5	G/A	20	1,453	0.94 (0.68-1.28)	0.68
rs7719391	*PCDH1*	5	G/C	31	1,452	0.93 (0.71-1.21)	0.58
rs11167761	*PCDH1*	5	G/A	18	1,454	0.80 (0.57-1.12)*	0.19
rs17097812	*PCDH1*	5	G/A	11	1,454	0.71 (0.46-1.11)*	0.13
rs3918396	*ADAM33*	20	G/A	9	1,453	1.32 (0.90-1.94)*	0.15
rs597980	*ADAM33*	20	G/A	25	1,453	1.11 (0.87-1.42)	0.41
rs2787094	*ADAM33*	20	G/C	25	1,452	1.08 (0.82-1.42)	0.59

MAF = minor allele frequency. [1] Adjusted for sex. *IL4* = interleukin 4. *IL4R* = interleukin 4 receptor. *IL13* = interleukin 13. *CDH1* = E-cadherin. *PCDH1* = Protocadherin-1. *ADAM33* = A Disintegrin And Metalloproteinase Domain 33. * $p \leq 0.20$. Bold $p<0.05$.

TABLE 3.3 | Estimated associations between respectively the GRS, *in utero* exposure to maternal smoking and maternal asthma and asthma development.

	OR (95% CI)			
	Model 1	Model 2	Model 3	Model 4
GRS	1.19 (1.03-1.36)*	NA	NA	1.25 (1.06-1.47)*
In utero exposure to smoking	NA	1.64 (1.15-2.33)*	NA	1.86 (1.23-2.80)*
Maternal asthma[1]	NA	NA	4.61 (2.95-7.19)*	4.63 (2.54-8.44)*

[1] At baseline (mean age 11 yrs). GRS = genetic risk score (rs3024647 (*IL4R*), rs3091307 (*IL13*), rs12447341, rs13689, rs8061932 (*CDH1*), rs14359, rs3822357, rs11167761, rs17097812 (*PCDH1*), rs3918396 (*ADAM33*)). NA = not applicable. * p<0.05.

TABLE 3.4 | Estimated interaction effect between GRS and common risk factors for asthma development i.e. *in utero* exposure to maternal smoking respectively maternal asthma, on asthma development

	OR (95% CI)	
	Model 1	Model 2
GRS	1.25 (1.02-1.53)*	1.18 (1.00-1.38)*
In utero exposure to smoking	2.66 (0.27-26.72)	NA
GRS* *in utero* exposure to smoking	0.94 (0.69-1.29)	NA
Maternal asthma[1]	NA	1.02 (0.04-24.28)
GRS*maternal asthma[1]	NA	1.23 (0.78-1.88)

[1] At baseline (mean age 11 yrs). GRS = genetic risk score (rs3024647 (*IL4R*), rs3091307 (*IL13*), rs12447341, rs13689, rs8061932 (*CDH1*), rs14359, rs3822357, rs11167761, rs17097812 (*PCDH1*), rs3918396 (*ADAM33*)). NA = not applicable. *p<0.05.

DISCUSSION

This study showed that a GRS based on the combined effect of SNPs in genes associated with Th2 cell differentiation and allergic inflammation, epithelial function and airway remodeling, is a highly significant independent predictor of asthma development. The predictive properties of the GRS were independent of the other risk factors, i.e. *in utero* exposure to maternal smoking and maternal asthma.

A single SNP in a single gene can only explain a small part of the variance in asthma prevalence. Therefore, combining these genes with modest effects into a genetic risk score (GRS) might result in a better prediction of asthma development. To our knowledge there are no previous studies that investigated the combined effect of *IL4R*, *IL13*, *CDH1*, *PCDH1* and *ADAM33* by constructing a GRS for asthma. We found that the GRS was associated with an increased risk to develop asthma, even after adjustment for well known risk factors for asthma development.

Not only genes, but also environmental factors and gene-environmental interactions are known to be involved in asthma development. Our study confirms previous studies showing that *in utero* exposure to maternal smoking is a risk factor for asthma development[12,13]. Exemplary, recent studies have shown *in utero* exposure to passive smoking seems to interact with *IL13*, *PCDH1* and *ADAM33,* resulting in an increased risk of early-onset persistent wheeze, excess lung function decline[14] and in the development of bronchial hyperresponsiveness[14-16]. However, our study suggests that the increased risk on asthma associated with a higher GRS was not dependent on *in utero* exposure to maternal smoking, since we observed no interaction between both on the risk of asthma development. Likewise, our study confirms results of previous studies that showed maternal asthma is a common risk factor for asthma development[16], and that there was no interaction between GRS and maternal asthma. However, since this is the first study investigating these interactions, further studies are needed to confirm our findings.

Our study shows that independent of common risk factors for asthma development (i.e. *in utero* exposure to maternal smoking and maternal asthma), our GRS is predictive for asthma development in adolescence. The question arises whether our current GRS is a better identifier of individuals at risk for asthma development compared to the common risk factors for asthma development that are easy to obtain and less expensive. To solve this question we compared the area under the receiver operating characteristic curve (AUC) to see which risk factor(s) would indentify best those individuals with asthma. Comparison of the AUCs revealed that the discriminative power to detect adolescents at risk for asthma was comparable for *in utero* exposure to maternal smoking, maternal asthma and GRS. Moreover, the discriminative power increased when information about *in utero* exposure to maternal smoking and maternal history of asthma were combined. However, additionally

adding GRS to this model did not clinically improve this discriminative power (Table E3.1 in online supplement). Although the discriminative power of our current GRS to detect adolescents at risk for asthma is comparable to common risk factors for asthma that are easier to obtain and less expensive, the GRS still is a useful tool since our study showed that the GRS is a highly significant independent predictor of asthma, even when *in utero* exposure to maternal smoking and maternal asthma are taken into account (Table 3.3). Moreover, knowledge of genetic background is necessary for unraveling (genetic) pathways underlying asthma development to reveal its etiology. In addition, by knowing these pathways one could start interventions to prevent asthma and develop medication to treat asthma exacerbations.

A strength of our study is its longitudinal design, allowing us to study asthma development using the GRS. Our study also has some limitations. There are more risk increasing genes for asthma development than only those belonging to the pathways selected in this study. In addition, there are also genetic variants in genes that seem to have a protective effect for the development of asthma. It would be of interest to test whether including SNPs of these genes improve the predictive value of GRS for asthma development.

In conclusion, the GRS can be used as a tool to predict development of asthma independent of common risk factors for asthma development such as *in utero* exposure to maternal smoking and maternal asthma. Its merits should be further explored on its potential in clinical and screening practice.

ONLINE SUPPLEMENT

Selection of SNPs

SNPs within genes associated with T helper 2 (Th2) cell differentiation and allergic inflammation (*IL4*: rs2243266, rs2227282, rs2243248, rs762534, rs2243263; *IL4R*: rs3024578, rs3024544, rs3024530, rs1805012, rs1805013, rs6498011, rs3024647, rs1110470, rs3024676, rs8832, rs2239347, rs1801275, rs2283563, rs4787956, rs8057585, rs4787948, rs3024622, rs12102586, rs3024613, rs3024585, rs2074570, rs2057768, rs3024560, rs1029489 and *IL13*: rs1295685, rs2243208, rs3091307, rs1295683, rs2243297), epithelial function (*CDH1:* rs9929218, rs11640099, rs1125557, rs12447341, rs13689, rs8061932, rs10431924, rs9927789, rs2011779, rs11646158, rs12597188, rs2276329, rs3931740, rs8056633, rs7188750, rs4783573, rs2276330, rs11075699, rs7203904, rs7186053, rs16958383 and *PCDH1*: rs6888135, rs14359, rs3935792, rs4382196, rs12515587, rs3822357, rs10054186, rs10063472, rs7719391, rs11167761, rs17097812) and airway remodeling (*ADAM33*: rs3918396, rs528557, rs597980, rs2787094, rs511898, rs612709, rs2280089) were selected based on previously shown associations with asthma in other studies[1-11] or tagging SNPs using Haploview.

Genotyping

At mean age 16 years (n=1,816), DNA was extracted from blood samples (n=1,207) or buccal swabs (Cytobrush®, n=258) using a manual salting out procedure as described byMiller and colleagues[19]. SNP typing was performed on the Golden Gate Illumina BeadStation 500 platform (Illumina, Inc., San Diego, CA, USA) following the manufacturer's protocol. Genotype clustering was performed in BeadStudio 3.0 (Illumina, Inc., San Diego, CA, USA). Of 1,454 adolescents, SNPs were genotyped.

Genotyping of rs1110470 (*IL4R*), rs1125557, rs11646158 (*CDH1*) and rs511898, rs612709, rs2280089 (*ADAM33*) and failed and were replaced by rs2040789 (*IL4R*), rs12448999, rs4783689 (*CDH1*) and rs17548913, rs17548907, rs2280091 (*ADAM33*) respectively. Genotyping of rs762534 (*IL4*), rs1801275, rs2074570 (*IL4R*), rs2011779, rs8056633, rs12597188 (*CDH1*), rs3797054, rs12515587 and rs3935792 (*PCDH1*) failed but could not be replaced by another SNP. Five SNPs were additionally excluded, three based on low call rates (rs2283563 (*IL4R*), rs7203904 (*CDH1*), rs528557 (*ADAM33*)) and two SNPs were not in Hardy-Weinberg Equilibrium (HWE p-value<0.000064 (=0.05/768); rs1805012, rs1805013 (*IL4R*)).

Call rates for the remaining SNPs were above 95%, were in Hardy-Weinberg Equilibrium (HWE) and all adolescents had less that 10% missing genotypes.

TABLE E3.1 | Area under the receiver operating characteristic curve (AUC) of models predicting asthma development

Model (Variable(s) included into the model)	AUC (95% CI)
Model 1 (GRS)	0.55 (0.50-0.60)
Model 2 (*In utero* exposure to maternal smoking)	0.56 (0.51-0.61)
Model 3 (Maternal asthma[1])	0.56 (0.52-0.61)
Model 4 (*In utero* exposure to maternal smoking, maternal asthma[1])	0.63 (0.58-0.69)
Model 5 (GRS, *in utero* exposure to maternal smoking, maternal asthma[1])	0.65 (0.59-0.71)

[1] At baseline (mean age 11 yrs). GRS = genetic risk score (rs3024647 (*IL4R*), rs3091307 (*IL13*), rs12447341, rs13689, rs8061932 (*CDH1*), rs14359, rs3822357, rs11167761, rs17097812 (*PCDH1*), rs3918396 (*ADAM33*)).

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CHAPTER 4

Stress exposure early in life is associated with asthma development. The TRAILS study

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Submitted.

ABSTRACT

Background: The role of stress in asthma development is still unclear. We examined (1) whether exposure to prenatal stress, postnatal stress or stressful life events before 4 years is associated with asthma development up to adolescence, and (2) whether perinatal stress (prenatal or postnatal stress) influence the relation between stressful life events before 4 years and asthma.

Methods: In the population-based TRacking Adolescents' Individual Lives Survey study, information about exposure to prenatal stress (maternal psychological problems during pregnancy), postnatal stress (maternal postnatal depression), stressful life events, and age of first asthma attack were retrospectively collected in 2,230 adolescents (51% female) at ages 11, 14 and 16 years. Hazard ratios (HR (95% CI)) were calculated for associations between stress exposures and asthma adjusted for sex and social-economic status.

Results: Prenatal stress exposure (2.32 (1.32-4.05)) and exposure to stressful life events before 4 years (1.42 (1.01-1.99)) were significantly associated with asthma development; postnatal stress (1.70 (0.90-3.21)) approached significance. Prenatal and postnatal stress exposure did not significantly increase the risk of asthma associated with subsequent stressful life events before 4 years (HRs of the interactions 1.68 (0.38-7.35) and 2.68 (0.57-12.48) respectively).

Conclusion: Our study suggests that exposure to prenatal stress and stressful life events experienced before 4 years may increase the risk of asthma development.

INTRODUCTION

Asthma is a chronic inflammatory airway disease with a multifactorial etiology. A known risk factor for asthma exacerbations is psychosocial stress[1,2]. However, there is still debate whether stress also contributes to the development of asthma. Several studies suggest a longitudinal association between specific types of stress and asthma development[3-11].

Studies performed in adults suggest an association between asthma development and family adversities experienced before the age of 18[3], breakup of relationships[4] or stressful life events[5]. The latter study, investigating the association between 10 stressful life events and asthma development, reports that especially illness of a family member, marital problems, divorce or separation and conflicts with a supervisor were associated with asthma development[5]. In contrast, a study performed in students aged 18-25 years did not find an association between stressful life events (severe disease or death of a family member and parental or personal conflicts) and asthma development[6].

Studies performed in children also showed an association between stress and asthma development[7-10]. These studies typically investigated asthma in children about age 7 in relation to maternally reported early life stressors such as prenatal maternal anxiety, parental difficulties in the first month of life and maternal distress from birth onwards[7-9]. One study reported an association between maternal intimate partner violence during the first 3 years of life with asthma at age 3[11].

None of the studies performed in children investigated the associations between exposure to stress and asthma development during the entire period from birth throughout adolescence, which is the age window at which most incident asthma cases occur. In addition, previous studies are mostly directed at one specific type of stress, often maternal problems occurring very early in life. It therefore remains unknown how stressors interact throughout the life course and add up to an increased risk of asthma. It is also unclear whether perinatal stress alters the effects of stress experienced early in life on the risk of asthma. Finally, it also remains unclear via which pathways stress induces asthma. The association between prenatal stress and asthma could for instance be explained by smoking during pregnancy or birth weight, given their suggested associations with both prenatal stress and with asthma development[12-15].

In a Dutch general population cohort of 2,230 adolescents, we studied the association between stress and asthma development. We investigated whether perinatal stress and exposure to stressful life events before 4 years are associated with asthma development, and whether this perinatal stress increases the risk of asthma associated with exposure to stressful life events before 4 years. Furthermore, we explored whether *in utero* exposure to maternal smoking, and birth weight explain the association between prenatal stress and asthma development.

METHODS

Study population and design

The TRacking Adolescents' Individual Lives Survey (TRAILS) is a prospective cohort study among Dutch adolescents. The TRAILS sample can be regarded as representative of adolescents in the general population. So far, three assessment waves have been completed: assessment wave 1 in 2001-2002 (n=2,230, mean ± SD age 11 ± 0.6 years), assessment wave 2 in 2003-2004 (n=2,149, mean ± SD age 14 ± 0.5 years) and assessment wave 3 in 2005-2007 (n=1,816, mean ± SD age 16 ± 0.7 years). Adolescents will be followed until at least the age of 24 years. Detailed information has been described elsewhere[16].

Asthma

Data on ever having asthma, doctor diagnosis of asthma, and age of first asthma attack were retrospectively reported by 1,615 adolescents at mean age16 years, using the standardized questionnaire of the European Community Respiratory Health Survey (ECRHS)[17]. In adolescents who did not fill in this questionnaire (n=615), information about asthma was defined as parental report of asthma at the mean age 11 or 14 years[18]. Asthma development was defined as the age of first asthma attack (reported in whole years).

Stress

Information about exposure to prenatal stress, postnatal stress and stressful life events before 4 years was obtained retrospectively in a non-standardized parent interview at adolescent's mean age 11 years. Well-trained interviewers visited one of the parents or guardians (preferably the mother, 95.6%) at their homes to administer an interview covering a wide range of topics[19]. Prenatal exposure to stress was defined as *in utero* exposure to self-reported maternal psychological problems. Postnatal exposure to stress was defined as self-reported maternal postnatal depression. Exposure to stressful life events before 4 years was defined as parental report of one or more stressful life events experienced by the child during the pre-school period. These stressful life events included death of a family member or another close person, parental divorce, and the child not living with their parents for at last three months. To assess the effect of exposure to stressful life events on asthma development, a sum score was calculated by adding up all stressful life events that the adolescent was exposed to. Individuals with a score of 0 were defined as not exposed, those with a score of ≥ 1 were defined as exposed.

In addition to the interview, the parent filled out self-report questionnaires, including the Depression Anxiety Stress Scales (DASS), a 21-item self-report questionnaire measuring depression, anxiety and feelings of stress in the past week. By summing scores for the relevant seven items, scores for depression, anxiety and stress were determined[20].

In utero exposure to maternal smoking and birth weight

Information about *in utero* exposure to maternal smoking was retrospectively reported by the parent at adolescent's mean age of 16 years using a standardized questionnaire[21]. Information about birth weight was retrospectively assessed in the parent interview at adolescent's mean age of 11 years. It has previously been shown in this cohort that parental recall of birth weight and smoking during pregnancy are reliable[22].

Social-economic status (SES)

Social-economic status (SES) was assessed at mean age 11 years using 5 indicators: family income (low income (less than €2,500), intermediate income (€ 2,500 to € 5,500) and high income (greater than €5,500)), educational levels of both parents (elementary education, lower tracts of secondary education, higher tracts of secondary education, senior vocational education, and university education), and occupational levels of both parents (low (elementary occupations, plant and machine operators and assemblers, craft and related trades workers), intermediate (skilled agricultural and fisheries workers, service workers, shop and market sales workers, clerks), high (technicians and associated professionals, professionals, legislators, senior officials, and managers)) using the International Standard Classification of Occupations (ISCO)[23]. We created a SES variable by averaging the indicators after standardization. The lowest 25%, intermediate 50%, and highest 25% of the scores were considered to represent low, intermediate and high SES, respectively[24].

Statistical analysis

Cox proportional hazard models were used to estimate risk of asthma development as a function of stress (*in utero* exposure to maternal psychological problems, maternal postnatal depression and exposure to stressful life events before 4 years) while adjusting for sex and SES. Because psychopathology of the informant could act as a confounder in the association between retrospective report of stress and asthma, Cox proportional hazard models estimating the effect of stress on asthma development were repeated while additionally adjusting for parental anxiety, depression and stress.

To study whether perinatal stress (*in utero* exposure to maternal psychological problems or exposure to maternal postnatal depression) increases the risk of asthma associated with subsequent exposure to stressful life events, interactions tests were performed. If these interaction tests were significant, Cox proportional hazard models were used to estimate whether a significant effect of stressful life events before 4 years on asthma occurred, stratified for perinatal stress while adjusting for sex and SES.

The third aim of our study was to explore whether prenatal risk factors for asthma (i.e. *in utero* exposure to maternal smoking or birth weight) explain the association between prenatal stress (*in utero* exposure to maternal psychological problems) and asthma development. To explore whether *in utero* exposure to maternal smoking explains the association between

prenatal stress and asthma development, Cox regression models were used adjusting for sex, SES and *in utero* exposure to maternal smoking. To explore whether birth weight explains the association between prenatal stress and asthma development, Cox regression models were used adjusting for sex, SES and birth weight.

For all models, the association between stress and asthma development was studied in the age window after the type of stress had occurred. All reported HRs are for the presence versus absence of the stressor.

Of the 224 adolescents reporting a history of asthma at the third assessment wave, age of first asthma attack was missing in 43. Age of first asthma attack in these adolescents was conservatively estimated by the age at the earliest report of asthma, which was the parent report at the first assessment wave (n=21) or the age at the third assessment wave (n=22)[18]. Adolescents who had not developed asthma were censored at their age at the third assessment wave. Adolescents who were lost to follow-up at the third assessment wave were censored at their age at the last assessment wave in which they participated, unless they had developed asthma before that age, in which case they were censored at the age at the earliest report of asthma, which was the parent report at the first (n=44) or second (n=7) assessment wave.

The assumption of proportional hazards was assessed by inspecting log-minus-log plots of the survival functions. All log-minus-log plots of the investigated stressors fulfilled the criterion of proportionality.

Statistical analysis were performed using SPSS Inc. Windows 18.0. P-values<0.05 (tested 2-sided) were considered to be significant.

RESULTS

Study population

Table 4.1 provides the summary characteristics of the study population. Of the 224 adolescents who reported a history of asthma at the third assessment wave, 209 (93%) reported that a doctor had diagnosed their asthma, 10 (5%) reported that their asthma was not diagnosed by a doctor, and in 5 (2%) information about doctor diagnosis was missing. Information about age at first asthma attack was available for 181 (81%) adolescents.

Of the group of 2,230 adolescents, 315 (14%) were exposed to one or more stressful life events during the pre-school period. Of these adolescents, 300 (95%) experienced one type of stressful life events, 14 (4%) experienced two and 1 (1%) experienced all three types of stressful life events.

TABLE 4.1 | Characteristics of the study population

Variable	Total population n = 2,230
Female	1,132 (51)
Asthma	224 (14)
Birth weight (g)	3,390 ± 617
In utero exposure to maternal smoking	479 (32)
First trimester	462 (31)
Second trimester	407 (27)
Third trimester	402 (27)
In utero exposure to maternal psychological problems	48 (2)
Exposure to maternal postnatal depression	50 (2)
Exposure to stressful life event before 4 years[1]	315 (14)
Death of family member or other close person	160 (7.2)
Parental divorce	157 (7.0)
Child not living with their parents for at last three months	14 (0.6)
Low social-economic status	553 (25)
Intermediate social-economic status	1,084 (50)
High social-economic status	551 (25)

All variables are expressed as number (percentage of non-missing values) or as mean ± SD. [1] Death of family member or other close person, parental divorce, and the child not living with their parents for at last three months.

In utero exposure to maternal psychological problems was associated with significantly higher exposure to maternal postnatal depression (postnatal depression present in 43 out of 2,120 (2%) mothers without *in utero* exposure to maternal psychological problems versus 7 out of 48 (15%) mothers with *in utero* exposure to maternal psychological problems (Chi-square=32.84, p<0.001)). Neither *in utero* exposure to maternal psychological problems nor maternal postnatal depression was significantly associated with exposure to stressful life events before 4 years (exposure to one of more stressful life events before 4 years in 300 out of 2,123 (14%) adolescents without *in utero* exposure to psychological problems versus 9 out of 48 (19%) adolescents with *in utero* exposure to psychological problems (Chi-square=0.82, p=0.37); exposure to one or more stressful life events before 4 years in 301 out of 2,123 (14%) adolescents without exposure to maternal postnatal depression versus 11 out of 50 (22%) adolescents with exposure to maternal postnatal depression (Chi-square=2.43, p=0.20)).

Association between *in utero* exposure to maternal psychological problems and asthma development from birth

In utero exposure to maternal psychological problems was positively associated with asthma development from birth (HR (95% CI) 2.32 (1.32-4.05), p=0.003) (Table 4.2). Adjusting for parental anxiety, depression and stress did not attenuate this association (2.40 (1.36-4.23)). Next, we explored whether prenatal factors explained the association between prenatal stress and asthma development from birth. We found that *in utero* exposure to maternal smoking slightly influenced the association between *in utero* exposure to maternal psychological problems and asthma development from birth (2.19 (1.11-4.29)). Birth weight did not attenuate the association between *in utero* exposure to maternal psychological problems and asthma development from birth (2.44 (1.39-4.27)).

Association between exposure to maternal postnatal depression and asthma development from the age of 1 year

The association between exposure to maternal postnatal depression and asthma development from the age of 1 year was positive, approaching significance (1.70 (0.90-3.21), p=0.100) (Table 4.2). Adjusting for parental anxiety, depression and stress did not attenuate this association (1.74 (0.91-3.30)).

Association between exposure to stressful life events before 4 years and asthma development from the age of 4 years

Exposure to stressful life events before 4 years was positively associated with asthma development from the age of 4 years (1.42 (1.01-1.99), p=0.046) (Table 4.2). Adjusting for parental anxiety, depression and stress did not attenuate this association (1.51 (1.06-2.14)).

Association between exposure to stressful life events before 4 years and asthma development from the age of 4 years in adolescents with and without exposure to perinatal stress (*in utero* exposure to maternal psychological problems or exposure to maternal postnatal depression)

In utero exposure to maternal psychological problems did not significantly increase the risk of asthma associated with exposure to stressful life events before 4 years (HR (95% CI) of the interaction between *in utero* exposure to maternal psychological problems and stressful life experienced before 4 years 1.68 (0.38-7.35)). Exposure to postnatal depression did not significantly increase the risk of asthma associated with exposure to stressful life events before 4 years (HR (95% CI) of the interaction between exposure to postnatal depression and stressful life events before 4 years 2.68 (0.57-12.48)).

TABLE 4.2 | Cox proportional hazard ratios (95% CI) of the association between stress exposure and asthma development adjusted for sex and SES

Type of stress	Sex-adjusted HR (95% CI)	Outcome	Overall population number	Number of asthma cases	Person-time overall (years)	Person-time by exposure (years)
In utero exposure to maternal psychological problems[1]	**2.32 (1.32-4.05)***	Asthma>0yrs	2,171	266	31,623	30,982 (0) 641 (1)
Exposure to maternal postnatal depression[1]	1.70 (0.90-3.21)	Asthma>1yrs	2,166	259	29,487	28,840 (0) 646 (1)
Exposure to stressful life events before 4 years[1,2]	**1.42 (1.01-1.99)***	Asthma>4yrs	2,133	212	23,260	19,956 (0) 3,305 (1)

SES = social-economic status. HR= hazard ratio. Reported HR are for the presence versus absence of the stressor. All HR are adjusted for sex and SES. *p-value<0.05. [1] Parental interview at adolescent's mean age 11 years. [2] Death of family member or other close person, parental divorce, and the child not living with their parents for at last three months. 0 = Not exposed. 1 = Exposed.

DISCUSSION

We found that *in utero* exposure to maternal psychological problems and exposure to stressful life events before 4 years are associated with asthma development up to adolescence. However, we found no evidence that perinatal stress increases the risk of asthma associated with exposure to stressful life events before 4 years. We found that *in utero* exposure to maternal smoking slightly influenced the association between *in utero* exposure to maternal psychological problems and asthma development from birth, however, birth weight did not.

The strength of this study is that it was performed in a representative sample of the general population, thereby increasing the probability that our findings are generalizable to the population at large. Moreover, we used different measures of stress exposure, including *in utero* exposure to maternal psychological problems, exposure to maternal postnatal depression, and exposure to stressful life events before 4 years. Thus, different types of stress, and different timings of stress were used in the analysis. Results on all stress measures were consistent with stress being a risk factor for asthma development, with particularly *in utero* exposure to psychological problems and exposure to stressful life events before 4 years being significant risk factors.

Our results with regard to the effect of exposure to prenatal stress and stress before 4 years on asthma development are in line with results from other studies showing that the risk of asthma is higher in children of mothers with high anxiety scores during pregnancy, children exposed to parental difficulties in the first month of life, to maternal intimate partner violence during the first 3 years of life, and to continued maternal distress (depression and anxiety) from birth until age 7[7-9,11]. None of the aforementioned studies investigated the effect of exposure to stress from conception until the age of 4 years on asthma development. In addition, these studies are mostly directed at one specific type of stress, often maternal problems occurring very early in life. It could be envisaged that stressors interact throughout one's life course and combine into an increased risk of asthma. We found nevertheless that exposure to prenatal stress and stressful life events early in life may independently contribute to asthma development, and that perinatal stress did not significantly increase the risk of asthma associated with exposure to stressful life events early in life any further. However, we can not fully exclude that there does not exist a cumulative effect, due to the relatively low numbers of asthmatics exposed to specific stressors. For instance, the HR (95% CI) of exposure to stressful life events before 4 years was 8.15 (1.17-56.87) in children with prenatal stress exposure compared to 1.39 (0.98-1.98) in children without prenatal stress exposure, and these HRs (95% CI) were 3.33 (0.70-15.77) in those with postnatal maternal depression compared to 1.37 (0.96-1.94) in those without.

So far, it was not established if stressful life events before 4 years are associated with childhood development of asthma. Thus, we present new evidence that, in addition to stress exposure *in utero*, exposure to stressful life events before 4 years may increase the risk of asthma development from 4 years up to adolescence.

The question arises which mechanisms may explain the associations between stress and asthma development. A recent study showed that prenatal maternal mood alters the methylation status of the human glucocorticoid receptor gene, leading to an altered hypothalamic-pituitary-adrenal axis (HPA axis) stress reactivity in the offspring[25]. Also cumulative chronic stress is able to alter HPA axis function[26]. These findings are of interest, since changes in HPA axis regulation influence inflammatory processes thereby possibly affecting asthma development[27].

The main limitation of our study is that it was based on retrospective self-report, leading to potential mood and recall biases. A major influence of mood bias is unlikely, since adjusting for parental anxiety, depression and stress at age 11 years of the child did not attenuate the associations between stress and asthma development. An indication for recall bias with regard to stressors is that prevalences of depression during pregnancy and postnatal depression in our study (2% and 2%, respectively) with those in the Dutch general population (10% and 8%, respectively)[28,29] are relatively low. Given the considerable lapse of time between the occurrence of the problems, it seems likely that especially severe cases are reported, although it could also be related to our question which only concerned the first month after delivery. Recall bias could also affect the report of the age at which the adolescent had the first asthma attack. However, validation studies of the questionnaire we used have shown excellent reliability over repeated assessments, while reliability was unaffected by either age of onset or by the difference between the age of onset and the age at interview[30]. Retrospectively reported age of onset of asthma has been also used in previous studies on this topic, with much wider retrospective time intervals (asthma onset from age 21 onwards, age of the cohort up to 98)[3]. Nevertheless, although our data provide indications that stress and asthma development are linked, a truly prospective study enrolling pregnant women and following their children for symptoms of asthma would be optimal to address this research question.

In summary, our study suggests that exposure to prenatal stress and stressful life events before 4 years are independent risk factor for asthma development. We could not find a cumulative effect of stress exposure on asthma development, which may be a true observation or the result from a still relatively small sample size of children with asthma development. Further prospective studies are needed to unravel the mechanisms via which stress leads to asthma development so that early intervention can take place to reduce the risk of asthma development.

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CHAPTER 5

Basal or stress-induced cortisol and asthma development: the TRAILS study

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ABSTRACT

Background: We examined the association between (1) cortisol levels and asthma or asthma development, and (2) cortisol levels upon stress and asthma. In addition, we performed a post hoc meta-analysis on results from the literature.

Methods: Cortisol, cortisol upon stress, asthma (doctor diagnosis of asthma and/or symptoms and/or treatment in the past 12 months) and asthma development (asthma at a specific survey while not having asthma at the previous survey(s)) were assessed in the TRAILS study (n=2,230, mean age at survey 1 11 years, survey 2 14 years and survey 3 16 years). Logistic regression models were used to study associations between (1) cortisol (cortisol awakening response, area under the curve (AUC) with respect to the ground (AUCg) or with respect to the increase (AUCi), and evening cortisol) and asthma or asthma development, and (2) cortisol upon stress (AUCg or AUCi) and asthma. The meta-analysis included nine case–control articles on basal cortisol in asthma.

Results: No significant association was found between (1) cortisol and asthma (age 11 years) or asthma development (age 14 or 16 years), and (2) cortisol upon stress and asthma (age 16 years). The meta-analysis found lower morning and evening cortisol levels in asthmatics compared to non-asthmatics; however, the summary estimates were not significant.

Conclusion: We found no evidence supporting a role for cortisol in asthma and asthma development.

INTRODUCTION

Asthma is a chronic inflammatory airway disorder, with many pathways involved in its aetiology. Psychosocial stress can trigger asthma exacerbations[1] and it is suggested that psychosocial stress is also involved in asthma development[2-6]. Psychosocial stress activates the hypothalamic–pituitary–adrenal (HPA) axis leading to an increase in cortisol secretion on top of the circadian rhythm of cortisol secretion[7]. Cortisol influences the activity of many systems in the human body, including the immune system[8]. Since cortisol induces a shift in the T-helper cell 1 (Th1)/Th2 balance of peripheral blood mononuclear cells towards a predominantly Th2 response[7], an alteration in HPA axis function is suggested to be one of the potential mechanisms via which psychosocial stress leads to asthma development.

Several cross-sectional studies have investigated cortisol levels in asthmatics and non-asthmatics, with diverse results. Not only have lower[9-11] or normal cortisol levels[12-16] been reported in asthmatics compared to non-asthmatics, but also higher[17,18] cortisol levels have been reported. Since all these studies investigated different aspects of the circadian pattern of cortisol secretion in relation to asthma, their results are incomparable.

Because psychosocial stress triggers asthma exacerbations[1], it could be argued that dysfunctions of the HPA axis become more evident under stressful conditions. One previous study investigated both basal cortisol levels and cortisol responses to a laboratory stress task (Trier Social Stress Test) in asthmatics and non-asthmatics[12]. Although this study found no differences in basal cortisol levels between asthmatics and non-asthmatics, cortisol levels in response to a laboratory stress task were lower in asthmatics compared to non-asthmatics, indicating hyporesponsiveness of the HPA axis which only became evident under exposure to stress. Because of the small sample size of this study (asthmatics n=17, non-asthmatics n=18) these findings need to be replicated in a larger sample size.

In conclusion, results from previous cross-sectional studies have suggested that asthmatics have an altered HPA axis function. However, it is unclear whether this alteration in HPA axis function precedes the development of asthma or is the result of asthma. To our knowledge, no previous longitudinal study has been performed to investigate alterations in HPA axis function as an aetiologic mechanism contributing to the development of asthma. We hypothesised that (1) there is a cross-sectional association between low cortisol and asthma, (2) low cortisol levels precede the development of asthma, and (3) adolescents with asthma have a blunted cortisol response upon exposure to stress.

MATERIALS AND METHODS

Study participants

The Tracking Adolescents' Individual Lives Survey (TRAILS) is a prospective cohort study among Dutch adolescents. A detailed description of the sampling procedure and methods has been published previously[19]. Briefly, the TRAILS target sample involved 10–12-year-olds (born between October 1, 1989 and September 30, 1990 for the first two municipalities or between October 1, 1990 and September 30, 1991 for the last three municipalities) living in five municipalities in the north of the Netherlands, including both urban and rural areas. A total of 135 primary schools were invited to participate, encompassing 3,483 eligible children. Of the 135 schools 13 refused to participate, resulting in the exclusion of 338 children. Of the 3,145 remaining eligible children, 210 were excluded because they were either unable to participate or incapable of participating due to severe mental retardation or due to a serious physical illness or handicap, or if no Dutch-speaking parent or parent surrogate was available (Turkish and Moroccan parents who were unable to speak Dutch were interviewed in their own language). After intensive recruitment efforts (including telephone calls, reminder letters and home visits), a total of 2,230 children (76.0%) were included in the study at baseline. So far, three assessment surveys have been completed (n=2,230, mean ± SD age 11 ± 0.6 years; n=2,149, 14 ± 0.5 years; n=1,816, 16 ± 0.7 years).

Cortisol collection at age 11 years

Cortisol was assessed from saliva taken at three times during one day: shortly after waking up (Cort07.00); 30 minutes after waking up (Cort07.30); and at 20.00 h (Cort20.00) at age 11 years. Saliva samples were received from 1,768 adolescents (details on collection and assays have been published previously[20]). Non-responders did not differ from responders in terms of sex; non-responders were slightly older (mean age 11.2 versus 11.1 years)[20].

Stress test and cortisol collection at age 16 years

At age 16 years, 715 adolescents performed a stress test, based on (but not identical to) the Trier Social Stress Task[21]. The stress test consisted of two parts. In the first part, the adolescents were instructed to prepare a 6-min speech about themselves and their lives and deliver this speech in front of a video camera. The speech was followed by a 3-min interlude in which the adolescents were not allowed to speak. In the second part, adolescents were asked to perform a 6-min mental arithmetic task. The adolescents were instructed to repeatedly subtract the number 17 from a larger sum, starting with 13,287. The mental arithmetic task was followed by a 3-min period of silence, after which the adolescents were debriefed about the experiment. Adolescents with a high risk of mental health problems were over-represented in this population (details in the online supplement). Cortisol was assessed from saliva collected prior to the stress test (Cort1), directly after (Cort2), and 20 minutes (Cort3) and 40 minutes (Cort4) after the stress test (details on collection and

assays have been published previously[22]). Non-responders did not differ from responders in terms of sex; non-responders were slightly older (mean age 16.4 versus 16.1 year)[23].

Asthma

Data on asthma were collected via questionnaires at age 11, 14 and 16 years[24]. Asthma was defined as having a doctor diagnosis (Did a physician give your child a diagnosis of asthma?) (assessed at age 11 years) and/or symptoms and/or treatment for asthma in the past 12 months (assessed at all surveys). Asthma development was defined as having asthma at a specific survey, while not having asthma at all previous surveys.

Statistical analysis

Area under the curve with respect to the ground (AUCg) (11 years), AUCg (stress-induced), area under the curve with respect to the increase (AUCi) (11 years) and AUCi (stress-induced) were calculated (formulae are in the online supplement)[20,25,26]. To correct for skewed distributions, cortisol values above or below 3 x SD of the mean were regarded as outliers and excluded, after which all cortisol values were transformed to z-scores to normalise the data in order to be able to compare results on different cortisol measures. Including adolescents with cortisol values below or above 3 x SD of the mean in the analysis did not change the results.

Logistic regression analyses were used to study associations between basal cortisol and asthma at age 11 years and between basal cortisol and asthma development at age 14 and 16 years. All analyses were adjusted for sex and quadratic effect of sampling month, since these are known to be associated with cortisol values[20] and/or asthma[24] in this cohort.

Furthermore, logistic regression analyses were used to study associations between cortisol response to stress test and asthma at age 16 years, adjusted for sex and sampling weights to correct for the oversampling of adolescents with a high risk of mental health problems, and in case of AUCi (stress-induced) also for baseline cortisol level (Cort1) (online supplement).

All analyses were repeated adjusting for depression (affective problems scale of the Youth Self-Report[27]), physical activity and smoking in case of basal cortisol, and additionally adjusting for age, body mass index (BMI) and oral contraceptive (OC) use in case of stress-induced cortisol. A previous study in this cohort showed that age and BMI were not related to basal cortisol[20].

To examine the impact of (inhaled and oral) steroid treatment on the above studied associations, sensitivity analyses were performed by excluding adolescents who used corticosteroid medication at age 11 years, in case of basal cortisol, or at age 16 years, in case of stress-induced cortisol. Statistical analyses were performed using SPSS Version 18.0 (SPSS Inc., Chicago, IL, USA). P-values<0.05 (tested two-sided) were considered to be significant.

RESULTS

Study population

Table 5.1 shows the characteristics of the study populations stratified according to asthma at age 11 years. 22 (1%) Adolescents used corticosteroid-containing medication. There was no significant difference in age, sex and cortisol levels between adolescents with and without asthma at age 11 years. However, adolescents with asthma used significantly more corticosteroid-containing medication compared to adolescents without asthma.

In 489 (30%) adolescents, the AUCi (11 years) was negative (cortisol levels measured at 07.00 h were higher compared to cortisol levels measured at 07.30 h). At age 11 years, no significant differences were found between adolescents with a positive or a negative AUCi with respect to the proportion of adolescents with asthma: 34 out of 474 (7%) adolescents with a negative AUCi had asthma versus 73 out of 1,122 (7%) adolescents with a positive AUCi had asthma (Chi-squared=0.24, p=0.63).

TABLE 5.1 | Characteristics of the study population stratified according to asthma at age 11 years

	Asthma n = 154 (8%)	No asthma n = 1,893 (92%)
Age years	11.2 ± 0.6	11.1 ± 0.6
Females	77 (50)	966 (51)
Cort07.00 nmol/l	11.7 (0.7-100)	10.9 (0.9-73.3)
Cort07.30 nmol/l	14.4 (2.0-100.0)	14.9 (0.2-131.0)
Cort20.00 nmol/l	1.5 (0.0-8.2)	1.7 (0.0-8.9)
AUCg (11 years)	6.6 (1.1-13.7)	6.6 (0.5-15.5)
AUCi (11 years)	0.8 (-3.2-4.8)	1.0 (-5.3-7.3)
Corticosteroid-containing medication	13 (11)*	9 (1)

Data are presented as number (percentage), mean ± SD or median (range). Cort07.00 = cortisol measured shortly after waking up. Cort07.30 = cortisol measured 30 minutes after waking up. Cort20.00 = cortisol measured at 20.00 h. AUCg = area under the curve with respect to the ground at age 11 years. AUCi = area under the curve with respect to the increase at age 11 years. * $p<0.05$.

Cross-sectional associations between cortisol levels at age 11 years and asthma

Logistic regression analyses, adjusted for sex and quadratic effect of sampling month, showed no significant cross-sectional associations between asthma at age 11 years and AUCg (11 years), AUCi (11 years) or Cort20.00 h (Table 5.2). Adjusting for depression, physical exercise and smoking did not essentially affect the results. In addition, excluding adolescents using corticosteroid-containing medication did not essentially affect the results.

TABLE 5.2 | Estimated OR (95% CI) of the association between basal cortisol measured at age 11 years and asthma or asthma development adjusted for sex and quadratic effect of sampling month

	Asthma at age 11 years	Asthma development at age 14 years	Asthma development at age 16 years
AUCg (11 years)	0.99 (0.80-1.23)	1.46 (0.97-2.19)	1.27 (0.76-2.12)
AUCi (11 years)	0.91 (0.73-1.12)	1.14 (0.74-1.76)	1.06 (0.63-1.76)
Cort20.00 nmol/L	0.89 (0.71-1.11)	1.26 (0.85-1.86)	1.12 (0.67-1.86)

Values are shown as z-scores. AUCg = area under the curve with respect to the ground at age 11 years. AUCi = area under the curve with respect to the increase at age 11 years. Cort20.00 = cortisol measured at 20.00 h.

Longitudinal association between cortisol levels at age 11 years and asthma development at age 14 or 16 years

Logistic regression analyses, adjusted for sex and quadratic effect of sampling month, showed no significant association between AUCg (11 years), AUCi (11 years) or Cort20.00, and asthma development at age 14 years, or age 16 years (Table 5.2).

Adjusting for depression, physical exercise and smoking did not essentially affect the results. In addition, excluding adolescents using corticosteroid-containing medication did not essentially affect the results.

Study population stress-induced cortisol

Table 5.3 presents the characteristics of the study population participating in the stress experiments, stratified according to asthma at age 16 years. 34 (5%) Adolescents used corticosteroid-containing medication. There was no significant difference in age, sex and cortisol levels between adolescents with and without asthma at age 16 years. Adolescents with asthma used significantly more corticosteroid-containing medication compared to adolescents without asthma.

Cross-sectional association between cortisol levels during the stress test and asthma at age 16 years

Logistic regression analysis, adjusted for sex and sampling weights to correct for the oversampling of adolescents with a high risk of mental health problems, showed no significant association between AUCg (stress-induced) and asthma at age 16 years (OR (95% CI) 0.94, (0.67–1.31)). Comparable results were found for the association between AUCi (stress-induced) and asthma at age 16 years (1.04 (0.74–1.47)). Adjusting for age, depression, physical exercise, smoking, BMI and OC use did not essentially affect the results. In addition, excluding adolescents using corticosteroid-containing medication did not essentially affect the results.

TABLE 5.3 | Characteristics of the population participating in the stress test according to asthma at age 16 years

	Asthma n = 42 (6%)	No asthma n = 621 (94%)
Age years	16.1 ± 0.6	16.2 ± 0.6
Females	23 (55)	315 (51)
Cort1 nmol/l	3.0 (0.3-10.8)	3.2 (0.0-70.1)
Cort2 nmol/l	4.3 (0.5-9.6)	3.8 (0.0-68.0)
Cort3 nmol/l	3.7 (1.1-11.8)	3.7 (0.0-55.5)
Cort4 nmol/l	2.8 (0.9-16.4)	3.3 (0.0-51.5)
AUCg (stress-induced)	226.3 (56.0-588.0)	234.0 (16.5-855.8)
AUCi (stress-induced)	36.5 (-244.0-372.5)	16.5 (-359.5-448.3)
Corticosteroid-containing medication	22 (52)*	11 (2)

Data are presented as number (percentage), mean ± SD or median (range). Cort1 = cortisol measured just before the stress test. Cort2 = cortisol measured direct after the stress test. Cort3 = cortisol measured 20 min after the stress test. Cort4 = cortisol measured 40 min after the stress test. AUCg = area under the curve with respect to the ground (stress-induced). AUCi = area under the curve with respect to the increase (stress-induced). * $p<0.05$.

DISCUSSION

The present study did not show an association between basal or stress-induced cortisol levels and asthma when analysing the data cross-sectionally. Furthermore, we found no association between basal cortisol levels and the development of asthma in the longitudinal analyses.

This is the first study that investigated associations between basal cortisol levels and asthma in a large study population (n=2,230 adolescents). We found no association between basal cortisol measured at age 11 years and asthma at age 11 years. Previous studies have shown lower[9-11], comparable[12-16] and higher[17,18] cortisol levels in asthmatics than non-asthmatics. Therefore, we have performed a post hoc meta-analysis to obtain a stronger conclusion about the association between cortisol and asthma. To allow pooling across studies that used different types of HPA axis measurements, we calculated a standardised mean difference with 95% confidence intervals of basal cortisol levels in the morning

and basal cortisol levels in the evening (Tables E5.1 and E5.2 in online supplement)[28]. Both morning and evening cortisol levels were lower in asthmatics than non-asthmatics; however, the summary estimates were not significant (Figures 5.1 and 5.2). Funnel plots of the morning and evening cortisol levels indicated a possible publication bias (Figures E5.1 and E5.2 in online supplement), suggesting unidentified unpublished articles, which are mostly negative or neutral articles. This potential publication bias would be compatible with the fact that our study, despite having the largest sample size published, failed to find any significant association between cortisol and asthma or asthma development. It again underlines the importance of publishing studies that report the absence of a significant association, especially those with large sample sizes.

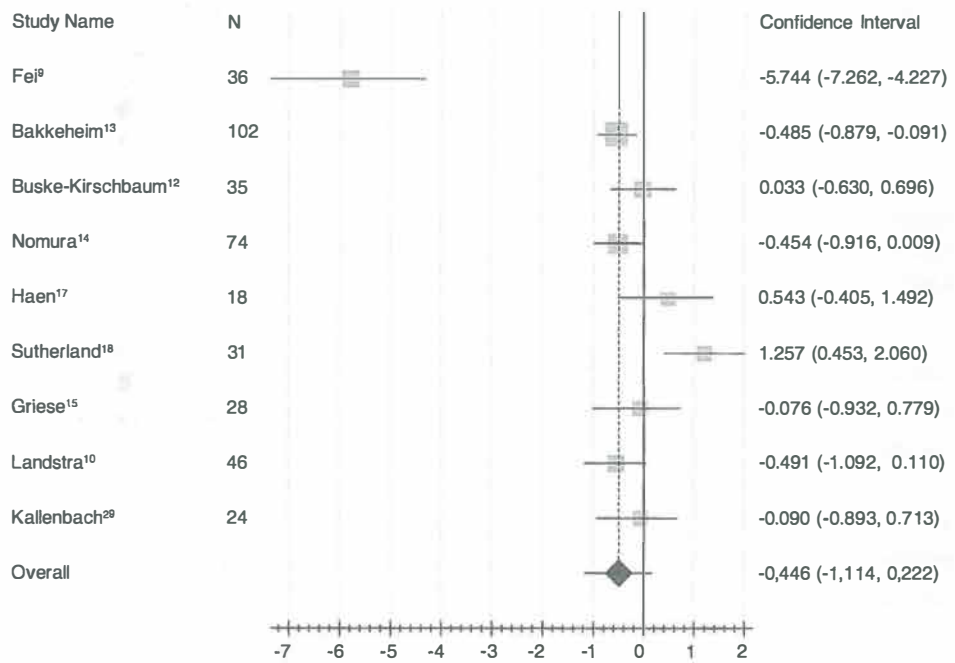

FIGURE 5.1 | Forest plot of the standardized mean differences morning cortisol levels in asthmatics and non-asthmatics. Test for heterogeneity Q-test Chi-square=70.04. Degrees of freedom=8, $p<0.0001$. $I^2 = 0.89$.

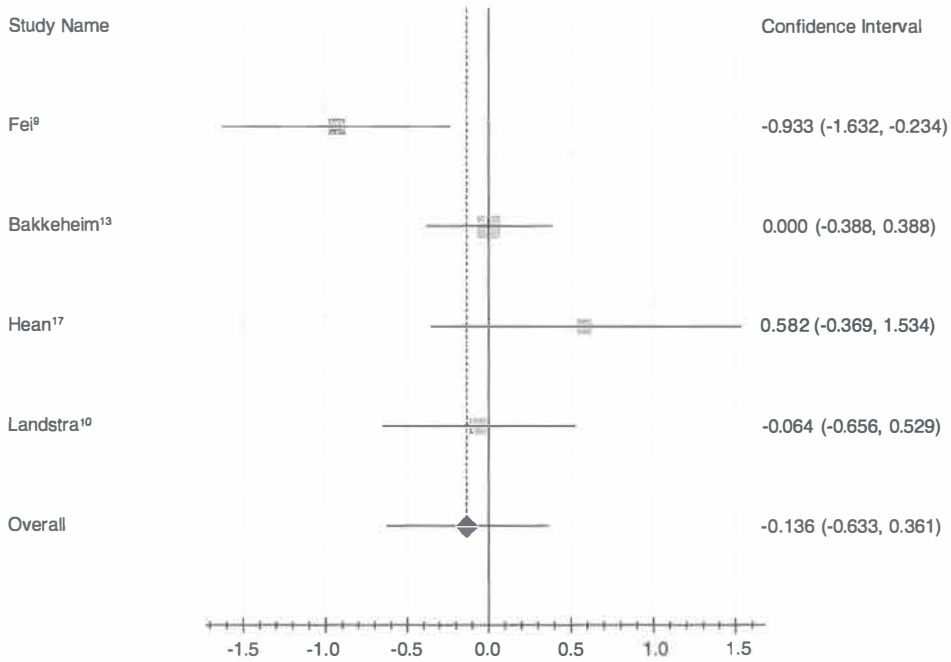

FIGURE 5.2 | Forest plot of the standardized mean differences evening cortisol levels in asthmatics and non-asthmatics. Test for heterogeneity Q-test Chi-square=7.70. Degress of freedom=3, p=0.05. $I^2 = 0.61$.

This study is the first one investigating the research question of whether alterations in HPA axis function precede asthma development. Neither asthma development at age 14 years, nor asthma development at age 16 years was significantly associated with basal cortisol levels upon awakening or evening cortisol level measured at age 11 years. Therefore, we did not find evidence that an alteration in HPA axis function precedes asthma development between ages 14 and 16 years. However, this study cannot rule out that alterations in HPA axis function precede adult-onset asthma.

Contrary to a previous study that showed a blunted cortisol response to stress in asthmatics[12], our study did not find such an association. Differences in study population and sample sizes could explain these inconsistent findings. The previous study included children (n=35, age 7–13 years), whereas our study included adolescents (n=715, age 15–17 years). Other explanations could involve differences in the time points at which cortisol was measured, and differences in the way the associations between cortisol levels to a stress task and asthma were studied. The previous study measured cortisol 35, 25, 15 and 1 min before, and 10, 20, 30 and 40 min after the stress test and compared individual time points between asthmatic and healthy children. Our study measured cortisol at the start,

directly after, and 20 and 40 min after the end of the stress test and investigated whether AUC was associated with asthma. When investigating individual cortisol levels measured at 20 and 40 min after the stress test in our study, we found no differences in cortisol levels between asthmatics and non-asthmatics (U=12,731.0, p=0.98 and U=11,453.0, p=0.39, respectively). Therefore, our data suggest that cortisol responses upon exposure to stress are not associated with asthma.

The strength of this study is its longitudinal design, allowing us to study the effect of cortisol prior to the onset of asthma. In addition, our study was performed in a large sample of the general population, thereby increasing the probability that our findings with respect to basal cortisol levels are generalisable to the population at large. However, this advantage of the large sample size comes with some associated disadvantages, especially the level of detail that was reached in the phenotyping of asthma and in the characterisation of HPA axis activity in our study. The asthma population in our study is heterogeneous in terms of phenotype, severity and management. Therefore, we may have missed potential associations with cortisol in specific subgroups such as those with nocturnal asthma or allergic asthma. We do not have information about whether adolescents with asthma have allergic or non-allergic asthma. However, we do have information about the presence of allergy, hay fever and eczema, which made it possible to give some indication whether asthmatic adolescents in our study have allergic (asthma with allergy, hay fever or eczema) or non-allergic asthma (asthma without allergy, hay fever and eczema). Sensitivity analysis revealed comparable associations with cortisol for allergic and non-allergic asthma (data not shown).

With respect to the characterisation of the HPA axis activity, cortisol was assessed at 1 day in our study. Previous studies showed that cortisol levels have notable intra-individual variability[12,16], which is reduced when sampling on a workday, as a result of the usually strict schemes[30]. In our study, cortisol was mostly assessed on school days, which are highly scheduled as well, so we expect that the same reduction in intra-individual variability applies to our adolescents. In addition, we expect that the large sample size will compensate for the possible reduction in reliability as a result of one day cortisol assessment, so random fluctuations in individual values will be set off.

In our study, cortisol was assessed at three time points, namely shortly after waking up (still lying in bed), 30 min after waking up and at 20.00 h. As a consequence only the cortisol awakening response and cortisol levels at 20.00 h could be studied in relation to asthma and asthma development, and not the circadian rhythm of the cortisol secretion. Therefore, we cannot rule out that there is an association between specific elements of this circadian rhythm of cortisol secretion and asthma presence or development.

We were unable to find evidence suggesting a role for cortisol in asthma, despite its well-established anti-inflammatory properties and therapeutic effects when given as inhaled or oral corticosteroids. The question arises whether our study indicates a real lack of association, or whether it merely reflects the problems associated with large cohort studies. Further prospective studies are needed to unravel the role of cortisol in asthma or asthma development. It is pivotal for such studies that both cortisol and asthma are measured in detail. For cortisol, this implies measuring multiple time points and multiple days, in order to obtain a reliable and robust estimate of HPA axis activity. For asthma, detailed phenotyping would enable the study of differences in the association with cortisol in relation to asthma severity and medication use, and also in relation to subtypes, including glucocorticoid resistant asthma or non-allergic asthma. Complaints of nocturnal worsening are especially interesting because of the circadian rhythm of cortisol secretion and the anti-inflammatory effect of cortisol.

In summary, our study did not find evidence supporting a role for cortisol in asthma and asthma development. In addition, our study found no association between stress-induced cortisol levels and asthma. Further longitudinal studies from birth onward have to assess whether there exists a window of time in which alterations in HPA axis function may contribute to asthma development. However, in adolescence, it seems unlikely that this plays a dominant role.

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ONLINE SUPPLEMENT

Selection of adolescents performing a stress test

The laboratory tasks started between 08.00 h and 09.30 h (morning sessions, 49%) and 13.00 h and 14.30 h (afternoon sessions, 51%). The costly and labor-intensive nature of the experimental session precluded assessing the whole sample. To increase the power to detect mental health-related differences in the stress response, adolescents with a high risk of mental health problems ha d a greater chance of being selected for the laboratory tasks session compared to healthy adolescents. High risk was defined based on three criteria: (1) temperament assessed by the revised parental version of the Early Adolescent Temperament Questionnaire, (EATQ; i.e., high scores on frustration ($\geq$ 90th percentile of EATQ), and fearfulness ($\geq$ 90th percentile of EATQ), low scores on effortful control ($\leq$ 10th percentile of EATQ)), (2) lifetime parental psychopathology (at least one parent with depression, anxiety, addiction, psychosis, or antisocial behavior), and (3) environmental risk (at least one of the biological parents was raised in a single-parent family), all assessed at age 11 years. In total, 66% had at least one of the above-described risk factors. The remaining 34% were randomly selected from the total TRAILS cohort. Please note that the focus sample still represented the whole range of problems seen in a normal population of adolescents, which made it possible to reproduce the distribution in the total TRAILS sample by means of sampling weights.

The study protocol was approved by the Central Committee on Research Involving Human Participants (CCMO).

Participants provided written informed consent. Forty-two (6%) adolescents had asthma, and 34 (5%) adolescents used corticosteroid-containing medication.

Stress task

The stress test consisted of two parts. In the first part, the adolescents were instructed to prepare a 6-minute speech about themselves and their lives and deliver this speech in front of a video camera. They were told that their videotaped performance would be judged by a panel of peers after the experiment. The adolescents had to speak continuously for the whole period of 6 minutes. The test assistant watched the performance critically, and showed no empathy or encouragement. The speech was followed by a 3-minute interlude in which the adolescents were not allowed to speak. During this interval, the adolescents were told that they had to wait for a moment because of computer problems, but that the task would continue as soon as the problems were solved. In the second part, adolescents were asked to perform a mental arithmic task. The adolescents were instructed to repeatedly subtract the number 17 from a larger sum, starting with 13,287. This difficult task was meant to induce a sense of uncontrollability. Uncontrollability was further provoked by negative feedback by the test assistant, including remarks such as, "No, wrong again, begin at

13,278", "Stop wiggling your hands" or "You are too slow, be as quick as you can, we are running out of schedule". The mental arithmetic challenge lasted for 6 minutes, again followed by a 3-minute period of silence, after which the adolescents were debriefed about the experiment.

Statistical analysis

With respect to cortisol measured upon awakening at age 11 years, the AUCg (11 years) was calculated using the formula (Cort07.30 – Cort07.00)* 0.5 / 2 + 0.5 * Cort07.00. The AUCi (11 years) was calculated using the formula (Cort07.30 – Cort07.00) * 0.5 / 2.

With respect to cortisol measured during the stress test, the AUCg (stress-induced) was calculated using the formula: (Cort1 + Cort2)* 25/2 + (Cort2 + Cort3)* 20/2 + (Cort3 + Cort4)*20/2. The AUCi (stress-induced) was calculated using the formula (Cort1 + Cort2)* 25/2 + (Cort2 + Cort3)* 20/2 + (Cort3 + Cort4)*20/2 – Cort1*65.

Search strategy for the meta-analysis

Relevant articles were identified by searching the database of Medline. The following search string was used to identify articles: the first component consisted of asthma; the second component consisted of the terms hypothalamic-pituitary-adrenal axis, pituitary-adrenal and cortisol; the third component consisted of the terms human, children, adolescents, childhood, adulthood, adult, adults.

Extraction of cortisol data

Morning and evening mean cortisol levels and standard deviations from asthmatics and non-asthmatics were extracted from the case-control studies or in case of missing information where calculated for each study. When cortisol levels were divided in nocturnal and non-nocturnal asthma or in mild and moderate-to-severe asthma, a pooled mean cortisol level and a pooled standard deviation was calculated. One study was excluded from the meta-analysis because calculation of mean cortisol levels and standard deviation in asthmatics and non-asthmatics was not possible. Relevant articles were identified by searching the database of Medline (published between 2012 and 1985). The following search string was used to identify articles: the first component consisted of asthma; the second component consisted of the terms hypothalamic-pituitary-adrenal axis, pituitary-adrenal and cortisol; the third component consisted of the terms human, children, adolescents, childhood, adulthood, adult, adults. This resulted in 601 articles. Title and abstract of relevant articles were screened based on: (1) case-control studies; (2) measurement of asthma; (3) measurement of HPA axis. Articles written in another language than English, articles studying the effect of treatment on HPA axis function, articles studying HPA axis under provocation tests or stress (test), and clinical trials were excluded. Full text of 11 articles was acquired. In case of suspected duplicate reports of the same subjects the original authors were contacted to detect overlap of individuals. This resulted in the exclusion of 1 article. In addition, 1 article

was excluded because no basal cortisol levels could be calculated from the information provided in the study.

Statistical software used for pooling the data

Meta-analyst (http://www.medepi.net/meta/MetaAnalyst.html) was used for pooling of the data. The present of heterogeneity was calculated by the Q statistic (Chi-square test calculating whether variation in study results is due to chance variation or whether variation is due to systematic underlying differences and the null hypothesis should be rejected) and the I^2 statistic (the percentage of variability in the results that is caused by heterogeneity rather than coincidence).

Allergic and non-allergic asthma

Parentally reported information about allergy, hay fever and eczema was assessed at age 11, 14 and 16 years. Allergic asthma was defined as having asthma at a specific survey with allergy, hay fever or eczema at that survey. Non-allergic asthma was defined as having asthma at a specific survey without allergy, hay fever or eczema at that survey.

Logistic regression models were used to study the association of basal cortisol with allergic and non-allergic asthma, adjusted for sex and quadratic effect of sampling month. Logistic regression models were used to study the association of stress-induced cortisol with allergic and non-allergic asthma, adjusted for sex and sampling weights to correct for the oversampling on high risk of mental health problems in case of AUCg (stress test) and additionally for baseline cortisol level (Cort1) in case of AUCi (stress test).

TABLE E5.1 | Overview of studies included in the meta-analysis comparing standardized mean differences of morning cortisol levels

Study	Saliva/ Serum	Cases			Controls		
		n	Mean (ng/ml)	SD (ng/ml)	n	Mean (ng/ml)	SD (ng/ml)
Fei[9]	Saliva	21	4.0	0.8	15	9.1	1.0
Bakkeheim[13]	Saliva	50	3.1	1.7	52	3.9	1.6
Buske-Kirschbaum[12]	Saliva	17	7.8	3.0	18	7.7	3.1
Nomura[14]	Serum	35	46.2	19.3	39	56.4	25.0
Haen[17]	Serum	10	250.1	103.3	8	206.6	30.8
Sutherland[18]	Serum	20	155.3	37.7	11	107.0	39.8
Griese[15]	Serum	21	126.9	77.7	7	132.5	55.8
Landstra[10]	Serum	28	116.0	86.3	18	159.5	92.3
Kallenbach[29]	Serum	13	148.5	39.8	11	151.8	32.5

n = number. SD = standard deviation.

TABLE E5.2 | Overview of studies included in the meta-analysis comparing standardized mean differences of evening cortisol levels

Study	Saliva/ Serum	Cases			Controls		
		n	Mean (ng/ml)	SD (ng/ml)	n	Mean (ng/ml)	SD (ng/ml)
Fei[9]	Saliva	21	2.9	0.6	15	3.5	0.7
Bakkeheim[13]	Saliva	50	0.3	0.1	52	0.3	0.2
Hean[17]	Serum	10	54.4	34.4	8	32.6	41.0
Landstra[10]	Serum	28	32.6	95.9	18	38.1	69.2

n = number. SD = standard deviation.

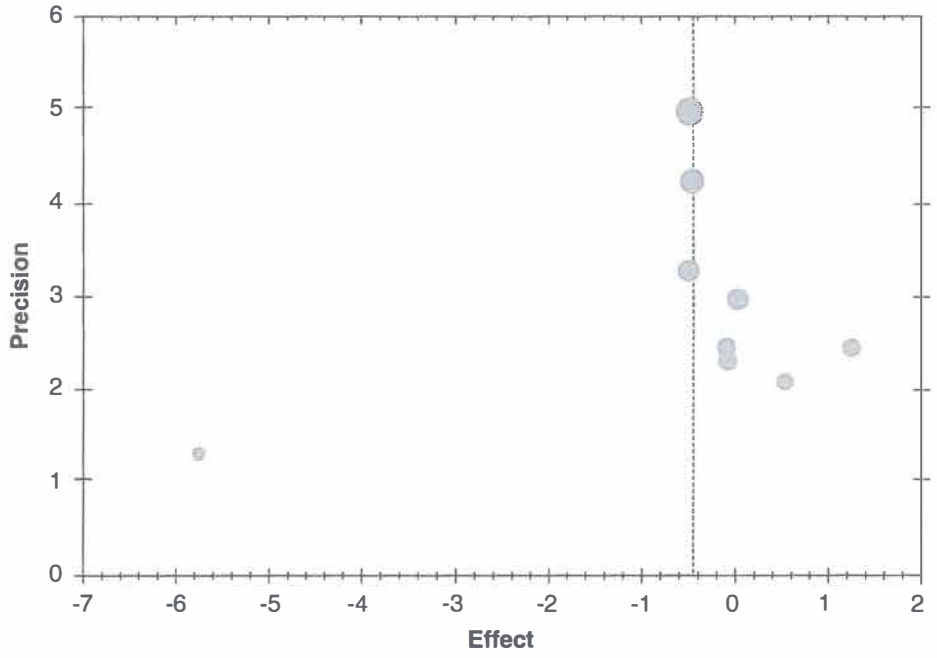

FIGURE E5.1 | Funnel plot of studies investigating morning cortisol levels in asthmatics and non-asthmatics.

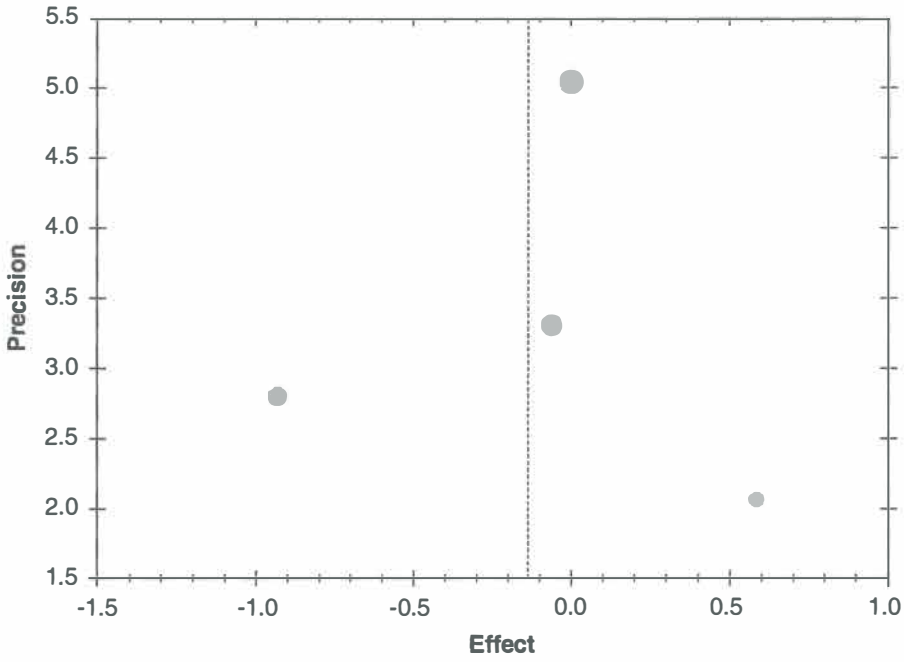

FIGURE E5.2 | Funnel plot of studies investigating evening cortisol levels in asthmatics and non-asthmatic.

CHAPTER 6

No associations of the mineralocorticoid and glucocorticoid receptor genes with asthma

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TO THE EDITORS:

Asthma is a multifactorial disease. Although a number of host and environmental risk factors have been identified in the past decades, these cannot fully explain the prevalence of asthma. Previous studies have shown that psychosocial stress confers a risk for the development of asthma[1].

Stress activates the hypothalamus–pituitary–adrenal (HPA) axis, which leads to secretion of cortisol. Cortisol in turn influences the activity of many systems in the human body and shifts, among others, the T-helper type (Th1/Th2) balance of the peripheral blood mononuclear cells towards a predominantly type 2 response. Cortisol binds to the high affinity mineralocorticoid receptor (*NR3C2*) and the low affinity glucocorticoid receptor (*NR3C1*). Single nucleotide polymorphisms (SNPs) in these receptors have been associated with basal cortisol and cortisol responses to stress[2]. So far, two studies have found an association between SNPs in *NR3C1* (i.e. rs6195, rs41423247) and asthma development[3,4]. However, these results have not been replicated in other populations so far. Additionally, it is unknown whether other functional or tagging SNPs in the *NR3C1* are associated with asthma development. In addition, SNPs in the *NR3C2* have not been studied before in relation to asthma development. Also it is yet unclear whether exposure to psychosocial stress modifies the effect of SNPs in *NR3C2* or *NR3C1* on asthma.

We investigated the associations of SNPs in *NR3C2* or *NR3C1* with asthma in adolescents from the general population and attempted to replicate our findings in an asthma case–control study.

In adolescents from the general population, we have previously shown that exposure to perinatal stress more than doubles the risk of asthma development[5]. Therefore, we tested additionally whether exposure to perinatal stress modifies the effect of SNPs in *NR3C2* or *NR3C1* on asthma in adolescents.

We genotyped two functional SNPs in *NR3C2* (rs5522, rs2070951) and 12 functional or tagging SNPs in *NR3C1* (rs4912903, rs6198, rs6196, rs258813, rs33388, rs17100236, rs10482642, rs2963155, rs41423247, rs9324924, rs4244032, rs4607376) in 1,454 adolescents of the prospective TRacking Adolescents' Individual Lives Survey (TRAILS) cohort (48% males; mean ± SD age at survey 1, 11 ± 0.6 yrs; survey 2, 14 ± 0.5 yrs; survey 3, 16 ± 0.7 yrs)[6] and in 998 individuals from the Dutch Asthma GWAS study (44% males; mean ± SD age 42 ± 12.2 yrs)[7], that acted as replication study. In TRAILS, data on parentally reported asthma was collected at age 11, 14 and 16 yrs via self-reported questionnaires[8]. Asthma was defined as a doctor diagnosis of asthma, and/or symptoms of asthma and/or

asthma treatment prescribed by a physician in the past 12 months, before or at the age of 16 years (n=141 (10%)). Adolescents not meeting these criteria were defined as not having asthma (n=1,293 (89%)). Information about asthma was missing in 20 adolescents (1%). The replication study consisted of 529 asthmatics and 469 non-asthmatics; asthma was defined as a doctor's diagnosis of asthma, the presence of asthma symptoms and bronchial hyperresponsiveness[7]. In TRAILS, perinatal stress was defined as *in utero* exposure to the mother's self-reported maternal psychological problems and/or self-reported maternal postnatal depression (n=67 adolescents (5%))[9].

SNPs were analysed in an additive genetic model for the effect on asthma using logistic regression models. Interactions between genotype and perinatal stress were tested in TRAILS by introduction of perinatal stress and the interaction term of genotype times perinatal stress into the model.

None of the SNPs in *NR3C2* or *NR3C1* were significantly associated with asthma in adolescents. We also found no association between these SNPs in *NR3C2* or *NR3C1* and asthma in the replication study (Table 6.1).

We observed no effect of perinatal stress on the association between SNPs in *NR3C2* or *NR3C1* and asthma in adolescents (i.e. no interaction between perinatal stress and SNPs).

Previous studies have shown that SNPs in *NR3C1* (i.e. rs6195, rs41423247) were associated with asthma[3,4]. Our results do not support the findings of these previous studies, specifically not those suggesting that a mutation (G/C) within rs41423247 in *NR3C1* would have a protective effect on asthma development[4]. However, this association was shown in merely one study including only 59 adults with asthma and 70 healthy adults. Since we found neither an association between rs41423247 and asthma in 1,434 adolescents (OR (95% CI) 0.99 (0.76–1.28)) nor in 998 adult individuals (0.96 (0.80– 1.15)), we feel that the findings from the study of *PIETRAS et al.*[4] may be due to chance.

Although we found no association between SNPs in the glucocorticoid or mineralocorticoid receptor and asthma, this does not exclude a role of glucocorticoid or mineralocorticoid receptor activity in asthma development, since this activity is influenced by multiple other mechanisms, such as chaperone proteins that modulate the translocation of these receptors and post-translation changes that modify the conformation of these receptors. In addition, methylation of both receptors can also influence whether cortisol levels are associated with inflammatory processes[10].

TABLE 6.1 | Estimated associations between single nucleotide polymorphisms in *NR3C2* or *NR3C1* and asthma in the TRacking Adolescents' Individual Lives Survey (TRAILS) cohort study[#] and the Dutch Asthma GWAS (DAG) study

	TRAILS cohort[¶]		DAG study[+]	
	MAF (%)	OR (95% CI)	MAF (%)	OR (95% CI)
		NR3C2 (chromosome 4)		
rs5522	G (13)	0.88 (0.60-1.29)	C (14)	1.24 (0.96-1.60)
rs2070951	G (49)	1.27 (0.98-1.63)	G (53)	0.93 (0.78-1.10)
		NR3C1 ***(chromosome 5)***		
rs4912903	A (40)	0.92 (0.71-1.18)	T (43)	0.96 (0.81-1.14)
rs6198[§]	G (16)	0.87 (0.62-1.22)	G (18)	0.84 (0.67-1.06)
rs6196[ʃ]	G (15)	1.12 (0.80-1.56)	C (16)	0.90 (0.70-1.15)
rs258813	A (31)	0.99 (0.76-1.29)	A (33)	0.86 (0.72-1.04)
rs33388	T (47)	1.11 (0.86-1.42)	T (45)	1.15 (0.96 (1.36)
rs17100236	G (13)	1.14 (0.81-1.62)	C (11)	1.21 (0.91-1.60)
rs10482642	G (16)	0.87 (0.62-1.22)	C (18)	0.85 (0.68-1.07)
rs2963155	G (23)	1.21 (0.91-1.61)	G (22)	1.10 (0.88-1.37)
rs41423247[##]	C (37)	0.99 (0.76-1.28)	G (37)	0.96 (0.80-1.15)
rs9324924	A (33)	1.03 (0.80-1.33)	T (29)	0.97 (0.77-1.22)
rs4244032	G (20)	1.00 (0.73-1.37)	G (20)	0.87 (0.62-1.23)
rs4607376	G (50)	0.98 (0.77-1.26)	G (50)	0.98 (0.82-1.17)

[#] Information about asthma missing in 20 adolescents. [¶] n=1,434, 141 cases and 1,293 controls. [+] n=998, 529 cases and 469 controls. MAF = minor allele frequency. *NR3C2* = mineralocorticoid receptor gene. *NR3C1* = glucocorticoid receptor gene. [§] In DAG study genotyped with rs11740792 (r^2=1). [ʃ] In DAG study genotyped with rs9324918 (r^2=1). [##] In DAG study genotyped with rs853180 (r^2=0.965).

In conclusion, we show suggestive evidence that SNPs in *NR3C2* or *NR3C1* are not associated with asthma development. We also find no indications that perinatal stress would affect the role of these genes on asthma development. These results indicate that, while these genes are biologically plausible candidate genes for asthma development in relation to perinatal stress, they cannot be linked to the disease.

We are grateful to all adolescents, their parents and teachers who participated in this research and to everyone who worked on this project and made it possible.

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CHAPTER 7

General Discussion

The aims of this thesis were to unravel the paths from early life stress to the development of asthma up to adolescence, and to quantify the relative contribution of the mediating mechanisms via which stress leads to asthma development. We first focused on the role of pubertal development, and on the gender-related switch in asthma prevalence and the genetic pathways involved in asthma development. In the current chapter, all findings will be integrated and discussed, resulting in a new perspective on the role of psychosocial stress in asthma development.

MAIN FINDINGS OF THIS THESIS

In the TRAILS study, asthma prevalence at age 11 years was 8% for boys and 7% for girls. However, a gender switch occurred in asthma prevalence after age 11, resulting in a higher asthma prevalence in females (6%) than males (4%) at age 16 years. This gender switch in asthma prevalence could not be explained by pubertal stages.

Multiple genes (such as genes involved in allergy (*IL4*, *IL4R* and *IL13*), epithelial integrity (*CDH1* and *PCDH1*) or airway remodeling (*ADAM33*)) are known to be associated with asthma or asthma-related phenotypes. The effects of SNPs in these genes on asthma risk are mostly modest. Therefore, we assessed whether combining the effects of SNPs in these genes into a genetic risk score (GRS) might result in a better prediction of asthma development. Results from this thesis show that the GRS is a highly significant independent predictor of asthma, even when other risk factors for asthma development, such as *in utero* exposure to maternal smoking and maternal asthma, are taken into account. Moreover, the effect of the GRS on asthma development is not dependent on *in utero* exposure to maternal smoking or maternal asthma.

Next to genetic and environmental factors, also exposure to psychosocial stress is suggested to be associated with asthma development. Results from this thesis suggest that exposure to prenatal stress and stressful life events experienced before 4 years increases the risk to develop asthma. However, the mechanisms underlying this association are yet unclear. Exposure to psychosocial stress leads to an increase in cortisol secretion. Cortisol influences many systems in the human body, among others the immune system where it induces a shift in the Th1/Th2 balance towards a Th2 inflammatory response. Therefore, we hypothesized that changes in cortisol levels would be associated with asthma or asthma development. Results from this thesis show that there is no association between basal or stress-induced cortisol and asthma, indicating that the HPA axis functioning in asthmatic adolescents is not different from that in non-asthmatic adolescents in our cohort. Moreover, we found no significant association between basal cortisol and asthma development, indicating that cortisol changes do not precede the development of asthma

during this age span. Next, we also studied whether SNPs in genes associated with HPA axis functioning, i.e. *NR3C1* and *NR3C2*, are associated with asthma. In addition, we tested whether the association between SNPs in these genes and asthma were dependent on exposure to perinatal stress. We found no effect of SNPs in *NR3C1* or *NR3C2* on asthma. Moreover, exposure to perinatal stress did not influence these associations. Other plausible mechanisms that can underlie the stress-asthma association are health-risk behaviors, such as *in utero* exposure to maternal smoking and overweight. Results from this thesis suggest that *in utero* exposure to maternal smoking explains a small part of the association between exposure to psychosocial stress and asthma development, however, birth weight does not.

In the first section of the discussion, these main findings are interpreted and discussed while considering the possible methodological strengths and limitations of our studies. In the second part of the discussion, the clinical relevance of the thesis findings is discussed. The thesis will end with suggestions for further research to unravel the paths from early life stress to the development of asthma up to adolescence, and to quantify the relative contribution of the mediating mechanisms via which stress leads to asthma development.

PSYCHOSOCIAL STRESS AS RISK FACTOR FOR ASTHMA DEVELOPMENT

Evidence from both cross-sectional and longitudinal studies performed in children, adolescents and adults suggests that psychosocial stress is involved in the etiology of asthma[1-22] (Tables 1.1 and 1.2). However, there are still a number of important questions that remain to be clarified regarding the role of subjective experience of a stressor, the timing of the exposure to the stressor, modification of the effect of stress on asthma development by genetic susceptibility, and the mechanisms via which stress can lead to asthma development. In the following part of the discussion, these questions will be approached.

THE ROLE OF THE OBJECTIVE EXPOSURE TO A STRESSOR VERSUS THE SUBJECTIVE EXPERIENCE OF A STRESSOR ON ASTHMA DEVELOPMENT

For the effect of stress on human health, it is known that both the presence of a stressor (objective stress exposure) and the subjective experience of a stressor are important. Most previous studies investigated the role of objective stressors on asthma development[1,2,5-22]. These studies investigated a broad variety of stressors, ranging from exposure to a stressor

experienced by the subjects themselves (i.e. stressful life events, physical/sexual abuse or job stress) to exposure to a stressor experienced by others such as the parents (i.e. maternal anxiety or death of a child or spouse). Results from these studies mostly showed that exposure to an objective stressor is associated with an increased risk to develop asthma (Tables 1.1 and 1.2). Only two previous studies have investigated the role of the subjective experience of a stressor on asthma development, namely parental difficulties measured at the age of 3 weeks[3,4]. These studies found that the subjective experience of a stressor is associated with asthma development as well (Table 1.1). As far as we know, no previous study has investigated both the effect of objective exposure to stress and the effect of the subjective experience of stress on asthma development. Therefore, it was unknown whether the effect of stress on asthma development applies to both the objective stress exposure as well as the subjective experience of stress exposure. In **chapter 4**, we investigated whether objective exposure to stress, namely *in utero* exposure to maternal psychological problems, postnatal depression and stressful life events before 4 years, is associated with the development of asthma up to adolescence. Moreover, we also explored whether the subjective experience of stress ("How many unpleasant events did you experience between the age of 0 and 5 (or 6 and 11) years", ranging form 0 ("no unpleasant events experienced") to 10 ("very many unpleasant events experienced") is associated with asthma development. We found that *in utero* exposure to maternal psychological problems and exposure to stressful life events before 4 years are significantly associated with asthma development up to adolescence. In addition, we found a positive association between exposure to postnatal depression and asthma development up to adolescence, although this effect was of borderline significance (Table 4.2). These results indicate that psychosocial stress exposure early in life is associated with asthma development. Next, we explored the effect of the subjective experience of stress on asthma development. We found that the experience of unpleasant events between the ages 0-5 years is associated with asthma development from the age of 6 years (HR (95% CI) 1.28 (0.94-1.75)), although this association is borderline significant (p=0.12). No association was found between the experience of unpleasant events between the ages 6-11 years and asthma development from the age of 12 years (1.27 (0.66-2.46)) (data not shown). These results suggest that there might be an effect of the subjective experience of stress, at least at young ages, on asthma development. Although both the objective and the subjective stress measures showed an effect of stress on asthma development, the effect on asthma development was stronger for the objective stress measure than for the subjective stress measures. This might be due to the fact that the time window in which the stress measures were assessed differed between the objective stress measure (0-3 years) and the subjective stress measures (0-5 and 6-11 years). However, the most plausible explanation for the fact that the effect on asthma development differed between the objective and subjective measures is that they assess different types of stress exposure. The objective stress measure assessed exposure to stressful life events such as death of a family member, death of another close person, parental divorce and the child not living with their parents for at last three months.

A sum score was calculated by adding up all stressful life events that the adolescent was exposed to in the first 4 years of life, and adolescents with a sum score of 0 were defined as not exposed, whereas those with a sum score of ≥1 were defined as exposed. The subjective stress measure assessed exposure to unpleasant events (between 0-5 and 6-11 years). Adolescents filled in a Likert linear analog scale ranging from 0 ("no unpleasant events experienced") to 10 ("very many unpleasant events experienced"). In this measure, adolescents with a score of 0 were defined as not exposed, whereas those with a score of ≥1 were defined as exposed. In the latter measure, no information was available about the exact nature of the unpleasant events the adolescents were exposed to. Thus, not only severe life events but also less severe life events or daily problems could be incorporated in this measure. In addition, the personality of the adolescent will have played an important role in perceiving how unpleasant an event was.

Results from previous studies support our hypothesis that the risk to develop asthma is increased with exposure to life events that are of more severe nature. These studies found that especially physical or sexual abuse[7,16,18], violence[8,10-13,18,19], illness or death of a family member[14,18], divorce/breaking off a life partnership[14,21], and war-related stress[17] were associated with an increased risk to develop asthma.

CRITICAL PERIOD MODEL VERSUS THE ACCUMULATION OF RISK MODEL

Two main pathways have been identified via which early life experiences can affect health later in life, namely the critical period model and the accumulation of risk model. The critical period model underlies the fetal origins of the adult disease hypothesis, whereas the accumulation of risk model assumes that the effects of stress accumulate gradually over time. In **chapter 4**, we investigated which of the above mentioned models can be applied to the stress-asthma association. We found that *in utero* exposure to maternal psychological problems and exposure to stressful life events before 4 years are significantly associated with asthma development up to adolescence. In addition, we found a positive association between exposure to postnatal depression and asthma development up to adolescence (borderline significance; p=0.10). When investigating the effect of stress exposure during these specific time points on asthma development, we found that exposure to stress during the prenatal period is associated with the highest risk to develop asthma. Moreover, we found that the risk to develop asthma as a result of exposure to stress decreases with the passage of time. This could indicate that indeed the critical period model fits the stress-asthma association; however, it could also be the result of the fact that different stress measures were used during different time periods, namely *in utero* exposure to maternal psychological problems (prenatal period), postnatal depression (postnatal period) and

stressful life events (early childhood). Previous studies have also investigated stress exposure during the perinatal period. Results from these studies are in line with our observations, namely that exposure to stress during the perinatal period is associated with asthma development. These studies typically investigated asthma development in relation to maternal anxiety during pregnancy[1], death of a child or a spouse during pregnancy[2], parental difficulties in the first month of life[3,4] and stressful life events during their first year of life[6] (Table 1.1). Therefore it seems plausible that the critical period model is the model that best fits the observed stress-asthma association.

Does the former summary imply that the accumulation of risk model does not fit to the stress-asthma association? This model assumes that the risk of asthma accumulates with stress exposure over time independent of the timing of the exposure. We tried to investigate whether this accumulation of risk model fitted to the stress-asthma association by investigating whether the effect of stress exposure during early life (i.e. stressful life events before age 4 years) on asthma development was increased in those adolescents that were exposed to perinatal stress (i.e. *in utero* exposure to maternal psychological problems or postnatal depression). We did not find an effect of exposure to perinatal stress on the association between stress exposure during early life and asthma development in our study (**chapter 4**). This indicates that the accumulation of risk model does not fit the observed stress-asthma association. However, it is known that not only the objective stress exposure, but also the subjective stress exposure is important for asthma development. Therefore we also explored whether the effect of the subjective experiencing of stress from birth onwards on asthma development was increased in adolescents that were exposed to stress during the perinatal period. We found that within adolescents exposed to prenatal stress, the risk to develop asthma as a result of exposure to unpleasant events experienced between ages 0-5 years is higher (OR (95% CI) = 2.44 (0.28-21.45)) than in adolescents not exposed to prenatal stress (1.23 (0.90-1.69)), although the HRs are not significantly different between both groups. However, this accumulative effect of stress exposure does not apply to exposure to postnatal stress (1.39 (0.27-7.19) versus 1.26 (0.92-1.73) respectively for adolescents exposed to postnatal depression and those not exposed) (data not shown). Moreover, we were not able to perform comparable analysis for unpleasant events experienced between ages 6-11 years, since all but one adolescent with perinatal stress who developed asthma did this before the age of 12 years. Overall, results from our study do not confirm the accumulation of risk model in the stress-asthma association. However, our data regarding the effect of subjective stress exposure between ages 6-11 years could also not fully exclude that there is no effect of accumulative stress exposure on asthma development since practically all adolescent with perinatal stress developed asthma at an early age. Further studies in larger groups are needed to investigate whether there is indeed an accumulative effect of stress on asthma development.

MODIFICATION OF THE EFFECT OF STRESS ON ASTHMA DEVELOPMENT BY GENETIC SUSCEPTIBILITY

A well-known model in psychiatry is the stress-vulnerability model, which basically states that stress may lead to psychiatric problems if an underlying vulnerability is present. Previous studies investigating children with a familial predisposition for asthma found that exposure to stress increases the risk to develop asthma[3,4,6]. However, it is not clear whether this effect of stress on asthma development was dependent on the genetic vulnerability to develop asthma, since these studies did not include children without a familial predisposition for asthma. In addition, if the effect of stress exposure on asthma development would be dependent on an underlying genetic predisposition, the genes underlying this effect are yet unknown.

We applied the stress-vulnerability model to stress-asthma association. In **chapter 4** we investigated the effect of stress exposure during the prenatal period (i.e. *in utero* exposure to maternal psychological problems), the postnatal period (i.e. postnatal depression) and early childhood (i.e. stressful life events before age 4 years) on asthma development. In addition, we explored whether the effect of prenatal and postnatal stress exposure on asthma development was modified by parental asthma. We showed that stress exposure during the perinatal period and early childhood increased the risk to develop asthma. However, our results did not support the hypothesis that the effect of stress on asthma development is dependent on parental asthma. Neither the interaction term between prenatal stress exposure and parental asthma nor that of postnatal stress exposure and parental asthma are significant (data not shown).

Next, we explored whether the genetic background of an adolescent modifies the effect of perinatal stress exposure (i.e. *in utero* exposure to maternal psychological problems and/or postnatal depression) on asthma development. Plausible genes that can modify the effect of stress on asthma development are genes known to be associated with asthma (such as *IL4*, *IL4R*, *IL13*, *CDH1*, *PCDH1* or *ADAM33*). Since the effect of a single SNP in these genes on asthma development are mostly modest, we investigated whether a genetic risk score (GRS), that combines the effects of these individual SNPs, is associated with asthma development to get a better prediction of the genetic contribution to asthma development (**chapter 3**). We found that a GRS is a strong predictor for asthma, even after correcting for other risk factors known to be associated with asthma development, being *in utero* exposure to maternal smoking and maternal asthma. Due to low numbers of adolescents exposed to perinatal stress, the modifying effect of perinatal stress (i.e. *in utero* exposure to maternal psychological problems and/or postnatal depression) on the association between GRS and asthma could not be studied.

Next to the genes mentioned above, there are other plausible genes that can modify the effect of stress on asthma development. Exposure to stress leads to an increase in cortisol secretion. Cortisol exerts his effects via binding to the glucocorticoid and the mineralocorticoid receptor. It is known that deficits in the activity of these receptors modify the effect of cortisol on end organs, e.g. the immunosuppressive effect in the lungs[23]. SNPs in both receptors (glucocorticoid receptor (*NR3C1*) and mineralocorticoid receptor (*NR3C2*)) have been associated with changes in basal cortisol levels, and additionally with changes in the HPA axis function induced by the Trier Social Stress Test[23-25]. Therefore, it might be likely that the effect of stress exposure on asthma development is modified by the *NR3C1* or *NR3C2*. In **chapter 6**, we tested whether SNPs in *NR3C1* or *NR3C2* influence asthma risk. In addition, we tested whether the effect of these SNPs on asthma development is dependent of exposure to perinatal stress. We found no association between SNPs in *NR3C1* and asthma in two independent large cohorts. In addition, also SNPs in *NR3C2* were not associated with asthma in our cohorts. Moreover, we found no effect of perinatal stress exposure on the association between SNPs in *NR3C1* or *NR3C1* and asthma. As stated before, the effect of SNPs on asthma development are mostly modest and by combining the effect of SNPs a better prediction of the genetic susceptibility of the *NR3C1* and *NR3C2* on asthma development is achieved. Therefore, further studies are needed with these SNPs combined in a GRS on asthma development in order to investigate the role of perinatal stress exposure on this association.

THE MECHANISMS UNDERLYING THE STRESS-ASTHMA ASSOCIATION

The previous section showed that exposure to psychosocial stress is a risk factor for asthma development. However, the mechanisms via which psychosocial stress leads to asthma development remain to be clarified. Stress exposure may exert its influences on many levels, making it difficult to disentangle the different mechanisms underlying this stress-asthma association, such as the stress systems (HPA axis and ANS) and health-risk behaviors. In this section, we discuss the role of the HPA axis and health-risk behaviours such as *in utero* exposure to maternal smoking as mediating mechanisms in the association between psychosocial stress exposure and asthma development.

The role of the HPA axis in the association between stress and asthma development

Most studies that investigated the role of the HPA axis use cortisol as measure for the functioning of the HPA axis. In these studies, cortisol is mostly collected in saliva, because it is a noninvasive stress-free sampling procedure that permits the monitoring of HPA axis functioning in an individual's natural environment[26]. Moreover, salivary cortisol values reflect the biologically active, unbound fraction of cortisol, and have a high correlation with serum

cortisol[27]. In humans, cortisol secretion follows a circadian rhythm, with a sharp increase prior to awakening, reaching a peak approximately half an hour later (cortisol awakening response (CAR)[28,29]), after which the secretion of cortisol steadily declines and is at its lowest during midnight. During exposure to psychosocial stress, the secretion of cortisol on top of this circadian secretion is increased.

Since cortisol can induce a shift in the Th1/Th2 balance towards a Th2 inflammatory response, the HPA axis can be one of the potential mechanisms via which psychosocial stress can lead to asthma development. A previous study in the TRAILS cohort investigated the association between stressful life events and cortisol levels (before, directly after, 20 minutes and 40 minutes after a stress test) in 715 adolescents at age 16 years[30]. This study showed that exposure to perinatal stress is associated with increased cortisol reactivity upon stress. In addition, this study found that exposure to adversities during ages 6-11 years was associated with overall higher cortisol levels, especially in those exposed to perinatal adversity, while exposure to adversities during ages 12-13 and 14-15 were associated with overall low cortisol levels. These results showed that there is an effect of stress on cortisol levels during adolescence which depends on the timing of the stress exposure. Therefore, it is plausible that changes in cortisol are responsible for the effect of stress on asthma. However, what is the direction of this association? Since increased cortisol levels lead to a predominant Th2 response of the immune system, one might expect that high cortisol levels are protecting against asthma. However, a recent review stated that a blunted HPA axis (characterized by lower morning cortisol levels and flattening of the diurnal slope) is associated with an increased susceptibility to develop autoimmune and inflammatory diseases[31]. Therefore, another hypothesis is that asthma is associated with lower cortisol levels. How this low cortisol levels lead to the development of asthma is yet unclear[31]. Results from previous cross-sectional studies did not consistently answer the question whether asthma is associated with higher or lower cortisol levels. These studies found lower[32-34], comparable[35-38] and also higher[39,40] cortisol levels in asthmatics compared to non-asthmatics. Therefore, we performed a meta-analysis on these studies to integrate previous findings on the association between cortisol and asthma. This meta-analysis revealed that morning and evening cortisol levels are lower in asthmatics compared to non-asthmatics, however, the summary effect estimates are not significant (Figures 5.1 and 5.2). Moreover, funnel plots of the included studies suggest the presence of publication bias (Figures E5.1 and E5.2 in online supplement). There was especially a lack of studies showing negative associations between cortisol levels and asthma in the literature. Thus, it still remains unclear whether there is an association between cortisol and asthma.

In **chapter 5**, we investigated the association between basal and stress-induced cortisol and asthma. In addition, we investigated the association between basal cortisol and asthma development in a longitudinal study design. Despite having the largest sample size published, our study did not find any significant association between basal or stress-induced

cortisol and asthma, which indicates that the HPA axis functioning in asthmatic adolescents is not different from that in non-asthmatic adolescents. Moreover, our study did not find any association between basal cortisol and asthma development. Several explanations can be given for the fact that we failed to find any significant association between cortisol and asthma or asthma development.

Heterogeneity in asthma phenotypes

The asthma populations described in previous cross-sectional studies were heterogeneous in terms of asthma phenotypes. Some studies used asthma as main outcome, and made no distinction between different asthma phenotypes, such as early onset allergic asthma/non-allergic asthma or nocturnal/non-nocturnal asthma. This is important because it is plausible that different mechanisms underlie different asthma phenotypes. For example, allergic asthma is associated with a Th2 response, which is less prominent in non-allergic asthma. Therefore, one could hypothesize that allergic asthma is associated with increased cortisol levels, whereas non-allergic asthma is not. Results from one previous study indicated that indeed allergic asthma was associated with lower cortisol levels[33]. This study showed that cortisol levels were lower in allergic asthmatics compared to healthy controls. However, most studies investigating individuals with allergic asthma did not find lower cortisol levels in allergic asthmatics compared to healthy controls, but found comparable[35,38] or even higher[39] cortisol levels in allergic asthmatics. Although the TRAILS study does not have detailed information about different asthma phenotypes, information about the presence of allergy, hay fever and eczema is available which might give an indication whether asthmatic adolescents in our study had allergic (asthma with allergy, hay fever or eczema) or non-allergic asthma (asthma without allergy, hay fever and eczema). In agreement with the literature, we found that cortisol levels are comparable in adolescents with allergic and non-allergic asthma. Nocturnal asthma is another phenotype of asthma that might be linked to lower cortisol levels, especially during midnight. Only one study previously investigated cortisol levels in nocturnal asthmatics, non-nocturnal asthmatics and healthy subjects[40]. This study found higher cortisol levels in individuals with nocturnal and non-nocturnal asthma compared to healthy ones. Further studies are needed, with detailed phenotyping of asthma, to investigate whether specific asthma phenotypes are associated with HPA axis alterations.

Next to detailed phenotyping of asthma, it is also important that these future studies take asthma severity into account. One previous study investigating 12 time points during a 24-hour period found that the acrophase in cortisol secretion (the increase in cortisol secretion) was delayed with 2 hours in adults with mild asthma and with 8 hours in adults with moderate-to-severe asthma[32]. Moreover, asthmatics, especially the more severe ones, are treated with corticosteroids. This is important since it is known that corticosteroid treatment lowers the endogenous cortisol production[41] via the negative feedback mechanism that regulates the cortisol production in the human body. Including asthmatics using corticosteroid

treatment into the study could thus confound the association between cortisol levels and asthma. Therefore, it is preferable to investigate asthmatics using corticosteroids separately from asthmatics not using this treatment when studying the association between cortisol levels and asthma. Most previous studies excluded asthmatics on corticosteroid treatment. These studies found lower[32,33], comparable[35,37] and higher[39,40] cortisol levels in asthmatics compared to non-asthmatics. A close comparison of these studies reveals that all studies used different time points at which their asthmatics were not allowed to take corticosteroid treatment, ranging from no treatment at all to no treatment with corticosteroids within the last 3 months prior to the start of the study. In **chapter 5**, where we studied the association of cortisol with asthma and with asthma development, we performed sensitivity analysis to investigate the confounding role of corticosteroid treatment on these associations. We found that excluding adolescents using corticosteroids did not essentially change our results.

Drawbacks of cortisol assessment in population based studies

Because cortisol secretion follows a circadian rhythm, a day-time cortisol profile is preferable over cortisol measures at one specific time point (i.e. the cortisol awakening response)[26]. Moreover, a recent review stated that a blunted HPA axis (characterized by lower morning cortisol levels and flattening of the diurnal slope) is associated with an increased susceptibility to autoimmune and inflammatory diseases[31]. This emphasizes the important role of collecting a day-time cortisol profile, since asthma is a chronic inflammatory disease. However, collecting a day-time profile is not always feasible because it is relatively expensive and time consuming. Previous studies all used different time points for collecting cortisol samples. Some studies collected a day-time cortisol profile (i.e. 12 time points during a 24-hour period, every 4 hour during a 24-hour period, every 2 hours during a 24-hour period or 4 samples during a 24-hour period at 2 days)[32,33,39,40], whereas others assessed cortisol in samples collected at specific time points (i.e. one time point, cortisol awakening response (CAR) on 3 consecutive days, one morning sample and one sample 1 hour after the last feeding or post-awakening and before bedtime)[34-38]. Thus, previous studies investigated different aspects of the circadian rhythm in relation to asthma development, which makes their results difficult to compare. In **chapter 5**, we tested whether basal cortisol levels (CAR and cortisol at 20.00 h) are associated with asthma or asthma development. We found no association between CAR and asthma or asthma development. Moreover, we found no association between evening cortisol and asthma or asthma development. One previous study investigated CAR in asthmatics and non-asthmatics[35]. Results from this study are comparable with our results, namely no differences in CAR between asthmatics and non-asthmatics. Two studies investigated the association between evening cortisol levels and asthma[36,38]. Although the timing of the evening cortisol samples differed between these two studies and our study, results from these two studies were comparable to ours, namely, that there was no association between evening cortisol levels and asthma. Based on these results, it seems that neither the CAR nor evening cortisol levels are associated with asthma. Altogether, studies so far could draw no conclusion on whether other aspects of the

circadian rhythm of cortisol secretion (e.g. a flattening of the diurnal slope) are associated with asthma. Studies assessing day-time cortisol profile in relation to asthma mostly studied a 24-hour mean cortisol level. These studies found lower[32,33] as well as higher[39] 24-hour mean cortisol levels. A close comparison of these studies reveals that cortisol samples were assessed at different time points in eac h study. Because cortisol secretion follows a circadian rhythm, measuring cortisol at different time points results in different cortisol levels which has consequences for the calculation of the 24-hour mean cortisol level in saliva or blood. In addition, also the amount of cortisol measures during this 24-hour period differed between these studies, and this has additional consequences for the 24-hour mean cortisol level in saliva and blood due to the circadian secretion of cortisol. This makes results from individual studies difficult to compare. Another study compared 12 time points during a 24-hour period (measured every 2 hours, started at 8.00 h) between non-nocturnal asthmatics, nocturnal asthmatics and healthy controls[40]. This study revealed that overall the cortisol levels in non-nocturnal and nocturnal asthmatics were higher compared to healthy controls. When comparing the individual time points, this study showed that non-nocturnal asthmatics had significantly higher cortisol levels compared to nocturnal asthmatics at 08.00 h and 10.00 h and at 4.00 h, 6.00 h, 8.00 h, 10.00 h and 12.00 h compared to the healthy controls.

In addition to the problems discussed above, cortisol levels are also influenced by many factors, including age, sex, BMI, depression, physical exercise, smoking and oral contraceptive use[26,27,42,43]. Studies that specifically adjusted for some of these variables, mostly sex and age[33,34,36], found lower[33,34] or comparable[36] cortisol levels in asthmatics versus non-asthmatics. In our study, we adjusted for all variables mentioned above and found that these variables do neither confound the association between basal or stress-induced cortisol and asthma, nor the association between basal cortisol levels and asthma development, suggesting that that the contradicting findings can not be explained by these variables.

The role of psychosocial stress in the cortisol-asthma association

One previous study investigating the association between asthma and cortisol levels in children age 7-10 years accounted for exposure to psychosocial stress[44]. This study found that cortisol levels were lower in asthmatic children compared to healthy children. However, this association was not significant. In addition, this study showed that exposure to maternal distress during the first year of life resulted in significantly higher cortisol levels. In comparison with children with no history of asthma or maternal distress, exposure to recurrent maternal distress resulted in higher cortisol levels in children without asthma, whereas in children with asthma, exposure to recurrent maternal distress resulted in lower cortisol levels. Moreover, within children with asthma, lower cortisol levels were found in children exposed to recurrent maternal distress than in those not exposed[44]. In this thesis, we found that exposure to stress, especially during the perinatal period and first years of life, is associated with an increased risk for asthma development (**chapter 4**). Although previous

studies suggest a role for cortisol in explaining the association between exposure to stress and asthma development, our study failed to find any significant association between cortisol and asthma or asthma development. Further studies that take exposure to stress during the perinatal period and early childhood into account should reveal whether cortisol levels are lower, especially in those adolescents with asthma that are exposed to stress during one or more of these periods.

The role of behavioural stress in the association between stress and asthma development

Exposure to psychosocial stress increases the likelihood of health-risk behaviors such as smoking and behaviors related to overweight (both eating and activity patterns). Exposure to psychosocial stress is associated with continuing smoking during pregnancy[45,46], a known risk factor for asthma development in the offspring[47]. Adolescents with a history of stressful events early in life are more likely to smoke regularly[48], which is another known risk factor for asthma[49]. Exposure to early life trauma is also associated with obesity later in life[50], again a risk factor for asthma[51]. Thus, smoking and overweight are potentially mediating factors in the relation between early life stress and asthma development. In **chapter 4**, we found that exposure to perinatal stress and exposure to stressful life events early in life are risk factors for the development of asthma. Moreover, we explored the role of *in utero* exposure to maternal smoking and birth weight as mediating factors in the association between psychosocial stress and asthma development. We found that *in utero* exposure to maternal smoking slightly decreases the HR of the association between *in utero* exposure to maternal psychological problems and asthma development from birth onward, indicating that a small part of the effect of prenatal stress exposure on asthma development is explained by *in utero* exposure to maternal smoking. However, we did not find an effect of birth weight on the association between prenatal stress exposure and asthma development. Whether other health-risk behavior factors are mediators in the association between exposure to early life stress and asthma needs to be investigated in further studies.

CLINICAL RELEVANCE

Results from this thesis show that prenatal stress exposure and exposure during early life are associated with an increased risk for asthma development. However, the mechanisms via which stress exposure leads to asthma development are not fully understood yet. Health-risk behaviors such as *in utero* exposure to maternal smoking appear to play a role in the association between stress exposure and asthma development.

Although stress exposure is not preventable, individuals can be made aware of the potential deleterious effect of stress on asthma development. Counseling programs may help in this

respect by focusing on awareness of the potential effects of stress and on ways to deal with stress exposure in order to reduce subjectively experienced stress and thereby possibly contributing to prevention of asthma development and to reduction of its severity. Such counseling programs might be targeted at pregnant women, midwifes, caregivers, teachers, children, adolescents and adults.

SUGGESTIONS FOR FUTURE RESEARCH

This thesis showed that exposure to stress during the perinatal period and early childhood is associated with asthma. However, some issues remain to be solved by future studies.

An ideal study should include women at the beginning of their pregnancy, and follow their offspring for a prolonged time. Next to already known risk factors for asthma development, such as air pollution, it is important to measure maternal exposure to psychosocial stress (both the objective exposure to stressors and the subjective experience of stress) and maternal mood and maternal smoking with validated questionnaires at multiple times during pregnancy. In addition, after birth, it is also important to measure psychosocial stress (objective and subjective stress) with standardized questionnaires at multiple times during the first years of life, since it is known that caregiving experiences during early childhood can influence the development of the HPA axis[52]. Exposure to stressful life events, asthma (including detailed information about specific phenotypes), atopy and life habits should be assessed preferably every year from birth onwards in these children, also using standardized questionnaires. With this information, the role of specific stressors on asthma development can be studied. Moreover, the questions whether there is a critical period for the effect of stress on asthma development, i.e. the perinatal period, and whether there is an accumulative effect of stress exposure on asthma development can be studied.

To study the role of the mediating mechanisms via which stress leads to asthma development, it is important to collect saliva multiple times during several days every year to determine cortisol levels as measure of HPA axis activity.

CONCLUSIONS

Historically, asthma has been defined as a psychosomatic disorder. However, with the understanding of the chronic inflammatory processes underlying asthma, researchers almost unanimously discarded the role of psychological factors in asthma development. In the last decade, the role of stress on asthma development has been reconsidered. Nowadays, psychosocial stress seems to be one of the risk factors next to genetic susceptibility and

environmental exposures that may contribute to asthma development. Results from this thesis showed that especially exposure to psychosocial stress in the perinatal period and early childhood is a risk factor for asthma development. Whether the effect of psychosocial stress exposure on asthma development is modified by the genetic make-up of an individual is yet not clear. Further studies are needed into this intriguing field of research to elucidate the role of stress as etiologic factor in asthma development.

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A D D E N D A

THESIS SUMMARY

The aims of this thesis were to unravel the paths from early life stress to the development of asthma up to adolescence, and to quantify the relative contribution of the mediating mechanisms via which stress leads to asthma development. We first focused on the role of pubertal development on the gender-related switch in asthma prevalence and risk factors for the development of asthma.

Chapter 1 describes asthma and the risk factors contributing to asthma development. One of these risk factors is exposure to psychosocial stress. The results of studies investigating the role of exposure to psychosocial stress on asthma development were discussed as well as the mechanisms via which exposure to psychosocial stress leads to asthma development. Additionally, the aims and the research questions of this thesis were described.

Chapter 2 describes the development of asthma up to adolescence. Specifically, the role of age and pubertal development on the gender-related switch in asthma prevalence were studied. In addition, the association between pubertal development and asthma-related phenotypes, such as total IgE and in peak expiratory flow (PEF) during a shuttle run test (SRT) were studied. This study showed that the prevalence of asthma is similar in boys and girls at a mean age of 11 years. The prevalence of asthma was significantly higher in female than male subjects at mean age 16 years. There were no significant associations between transition of pubertal stages and the presence of asthma, either cross-sectionally or longitudinally. Pubertal stages and total IgE levels or PEF fall during a SRT at age 16 years were not related. The conclusion of this study was that a shift in the prevalence of asthma occurs between age 11 and 16 years, which is due to both an increased incidence and decreased remission of asthma in female compared with male subjects.

In **chapter 3**, early life risk factors for asthma development were studied. These include the combined effect of SNPs in genes (genetic risk score (GRS)) involved in the development of asthma and asthma-related phenotypes on asthma development. The predicting properties of this GRS were compared to the predicting properties of known early life risk factors for asthma development such as *in utero* exposure to maternal smoking and maternal asthma. This study showed that both the GRS as well as *in utero* exposure to maternal smoking and maternal asthma were associated with asthma. Moreover, GRS remained significantly associated with asthma after adjustment for these common risk factors. There was no significant interaction between GRS and the common risk factors. The conclusion of this study was that the GRS is a highly significant independent predictor of asthma. Its merits should be further explored on its potential in clinical and screening practice.

In **chapter 4**, the role of exposure to perinatal stress and stress during early childhood on asthma development up to adolescence was studied. Results from this study show that *in utero* exposure to psychosocial stress and exposure to stressful life events (family member

or another close person, parental divorce, and the child not living with their parents for at last three months) before 4 years of age were significantly associated with asthma development up to age 16 years; exposure to postnatal stress approached significance. Moreover, we found that the risk to develop asthma as a result of exposure to stress decreased with the passage of time. In conclusion, this study showed that exposure to prenatal stress and stressful life events experienced before 4 years increased the risk of asthma development.

Chapter 5 and **6** focused on the role of the HPA axis on asthma and asthma development. In **chapter 5** the association between (1) cortisol levels and asthma or asthma development and (2) cortisol levels upon stress and asthma were studied. In addition, a meta-analysis was performed on results from the literature. Results from this study suggest no significant association between cortisol and asthma (age 11 years) or asthma development (age 14 or 16 years). In addition, no association was observed between level of cortisol upon stress and asthma (age 16 years). The meta-analysis showed there were lower morning and evening cortisol levels in asthmatics compared to non-asthmatics, however, the summary estimates were not significant.

In **chapter 6**, the association between SNPs in *NR3C2* or *NR3C1* and asthma development was studied in two independent cohorts. Additionally, interactions between genotype and perinatal stress were tested in TRAILS. Results from this study showed that there is no association between SNPs in *NR3C2* or *NR3C1* and asthma in two independent cohort studies. In addition, no significant interaction between genotype and exposure to perinatal stress was found.

In **chapter 7** the answers to the main research questions of this thesis were summarized. The results were discussed and methodological considerations and recommendations were made. In conclusion, this thesis showed that prenatal stress exposure and exposure during early life were associated with an increased risk for asthma development. However, the mechanisms via which stress exposure leads to asthma development are not fully understood yet. Health-risk behaviors such as *in utero* exposure to maternal smoking appear to play a small role in the association between stress exposure and asthma development. More research is needed that explores both physiological as well as behavioral stress effects as explanation for the link between stress and asthma.

NEDERLANDSE SAMENVATTING

Astma is een chronische ontstekingsziekten van de luchtwegen en longen. Iemand met astma is gevoelig voor prikkels waar gewone mensen niet gevoelig voor zijn, zoals bijvoorbeeld sigarettenrook, mist en huisstofmijt. Blootstelling aan deze prikkels zorgt voor een ontstekingsreactie in de luchtwegen wat resulteert in een variabele, meestal reversibele, luchtwegobstructie. Patiënten met astma hebben klachten als piepen, hoesten, benauwdheid en kortademigheid.

De laatste jaren is gebleken dat er een toename is van het aantal mensen dat astma ontwikkelt. Een genetische achtergrond kan dit niet makkelijk verklaren. Ook de reeds bekende omgevingsfactoren, zoals de blootstelling aan allergenen, sigarettenrook, huisstofmijt, of het hebben van een moeder met astma, kunnen deze toename van het aantal mensen dat astma ontwikkelt niet verklaren. Vandaar dat er de laatste jaren onderzoek wordt gedaan welke andere risicofactoren kunnen bijdragen aan het ontwikkelen van astma. Eén van de risicofactoren is de blootstelling aan psychosociale stress, de spanning die ontstaat wanneer iemand de eisen die de omgeving aan hem stelt niet meer aan kan.

In dit proefschrift wordt onderzocht of blootstelling aan psychosociale stress de kans op het ontstaan van astma vergroot. Tevens worden de mechanismen onderzocht die hierbij een rol zouden kunnen spelen. De onderzoeken in dit proefschrift maken deel uit van de TRacking Adolescents' Individual Lives Survey (TRAILS). Het TRAILS onderzoek is een grootschalig prospectief onderzoek waarin 2230 jongeren uit de drie noordelijke provincies van Nederland vanaf hun 10e tot hun 25e levensjaar gevolgd worden. Voor dit proefschrift werden de eerste drie metingen gebruikt waarin de kinderen gemiddeld 11, 14 en 16 jaar oud waren.

In **hoofdstuk 2** wordt de relatie tussen puberteitsstadia en geslachtsverschillen in de ontwikkeling van astma onderzocht. Uit dit onderzoek blijkt dat op 11- en 14-jarige leeftijd evenveel jongens als meisjes astma hebben. Echter op 14- en 16-jarige leeftijd hebben meer meisjes astma ontwikkeld, terwijl bij de jongens de astma klachten juist zijn verminderd. Vervolgens is onderzocht of geslachtsverschillen in het vóórkomen van astma parallel lopen met de verandering in puberteitsstadia tussen 11- en 16-jarige leeftijd. Daarnaast is onderzocht of puberteitsstadia op 16-jarige leeftijd samenhangen met astma gerelateerde kenmerken (totaal Immunoglobuline E (IgE) en een daling van de piekstroommeting (PEF) na een shuttle run test (SRT)). Resultaten van dit onderzoek laten zien dat er geen verband is tussen veranderingen in puberteitsstadia en het vóórkomen van astma. Ook is er geen verband gevonden tussen puberteitsstadia en totaal IgE of PEF na SRT. We concluderen uit deze studie dat geslachtsverschillen in de ontwikkeling van astma niet verklaard kunnen worden door puberteitsstadia.

In **hoofdstuk 3** wordt onderzoek gepresenteerd naar interleukine (*IL*) 4, *IL4* receptor (*IL4R*), *IL13*, E-cadherin (*CDH1*), Protocadherin-1 (*PCDH1*) en A DisintegrinAndMetalloprotease 33 (*ADAM33*). Single Nucleotide Polymorphisms (SNPs) in deze genen zijn in eerdere studies geassocieerd met astma of astma gerelateerde kenmerken, zoals het optreden van allergie, verstoorde epitheelcel-functie en toename in remodellering van de luchtwegen. Door het combineren van SNPs in een genetisch risico score (GRS) is het mogelijk een groter deel van het risico op het ontwikkelen van astma te kunnen verklaren. In **hoofdstuk 3** is de voorspellende waarde van de GRS voor het ontwikkelen van astma vergeleken met de voorspellende waarde van andere bekende risicofactoren van astma zoals roken gedurende de zwangerschap en het hebben van een moeder met astma. Daarna is onderzocht of genetische kwetsbaarheid, zoals gereflecteerd in de GRS, het effect van bekende risicofactoren op het ontwikkelen van astma beïnvloedt. Resultaten van deze studie laten zien dat de GRS, roken gedurende de zwangerschap en het hebben van een moeder met astma geassocieerd zijn met het ontwikkelen van astma. Na het corrigeren voor roken gedurende de zwangerschap en het hebben van een moeder met astma is de GRS nog steeds geassocieerd met het ontwikkelen van astma. Daarnaast is er geen significante interactie gevonden tussen de GRS en roken gedurende de zwangerschap of het hebben van een moeder met astma. Hieruit kunnen we concluderen dat het effect van GRS op het ontwikkelen van astma in adolescenten onafhankelijk is van roken gedurende de zwangerschap of het hebben van een moeder met astma.

In **hoofdstuk 4** wordt de relatie tussen blootstelling aan psychosociale stress en het ontwikkelen van astma onderzocht. Eerdere studies hebben aangetoond dat zowel de kans op het ontwikkelen van astma-exacerbaties als de kans op ziekenhuisopname ten gevolge van deze exacerbaties is vergroot na blootstelling aan psychosociale stress. Welke rol psychosociale stress in de ontwikkeling van astma speelt is minder duidelijk. Resultaten van deze studie laten zien dat blootstelling van de moeder aan psychosociale stress gedurende de zwangerschap de kans op het ontwikkelen van astma in het kind tot de leeftijd van 16 jaar vergroot. Deze kans is ook verhoogd na de blootstelling aan stressvolle gebeurtenissen in de eerste vier levensjaren (zoals het overlijden van een familielid, scheiding van de ouders en het niet thuis wonen gedurende tenminste 3 maanden). Het effect van blootstelling aan psychosociale stress op het ontwikkelen van astma wordt kleiner naarmate de leeftijd waarop de blootstelling aan psychosociale stress toeneemt. Vervolgens is in deze studie gekeken of roken gedurende de zwangerschap en geboortegewicht een deel van het verhoogde risico op het ontwikkelen van astma als gevolg van de blootstelling van de moeder aan stress tijdens de zwangerschap kan verklaren. Resultaten van dit onderzoek laten zien dat roken gedurende de zwangerschap inderdaad een klein deel van het effect van stress op het ontwikkelen van astma verklaart. Voor geboortegewicht is dit niet het geval.

In **hoofdstuk 5** wordt de rol van basaal cortisol in astma en astma ontwikkeling onderzocht. Bij stress nemen de concentraties van het stress-hormoon cortisol toe. Cortisol beïnvloedt onder andere het immuunsysteem en zou daarmee de kans op astma kunnen verhogen of verlagen. Het is nog steeds onduidelijk of astma nu gepaard gaat met een verhoogde, dan wel een verlaagde cortisolconcentratie in het bloed. Daarnaast is de rol die cortisol speelt in de ontwikkeling van astma nooit eerder onderzocht. Op de leeftijd van 11 jaar is onderzocht of cortisolconcentraties direct na het ontwaken geassocieerd zijn met astma. Daarnaast is onderzocht of cortisolconcentraties na een stress test afgenomen op 16-jarige leeftijd geassocieerd zijn met astma. Resultaten van dit onderzoek laten zien dat er geen verband is tussen cortisolconcentraties, zowel direct na het ontwaken als na de stress test, en astma. Vervolgens is onderzocht of cortisolconcentraties direct na het ontwaken op 10-jarige leeftijd geassocieerd zijn met het ontwikkelen van astma op 14- en 16-jarige leeftijd. Resultaten van dit onderzoek laten zien dat er geen verband is tussen cortisolconcentraties na het ontwaken en de ontwikkeling van astma. Om de vraag toch te beantwoorden of astma gepaard gaat met verhoogde of verlaagde cortisolconcentratie is een meta-analyse uitgevoerd op resultaten beschreven in de literatuur. Deze meta-analyse laat zien dat zowel de ochtend- als de avond-cortisolconcentraties lager zijn in mensen met astma ten opzichte van gezonde mensen, maar de verschillen zijn niet significant. De conclusie van de studie in ons cohort van jonge mensen rond de puberteit is dus dat er geen bewijs is dat cortisol een rol speelt in astma en de ontwikkeling van astma.

Hoofdstuk 6 onderzoekt SNPs in receptoren die cortisol binden, namelijk de mineralocorticoid en glucocorticoid receptor. Via binding aan deze receptoren beïnvloedt cortisol het immuunsysteem. SNPs in deze receptoren (mineralocorticoid receptor (*NR3C2);* glucocorticoid receptor (*NR3C1))* zijn geassocieerd met veranderingen in basale cortisol concentraties en met veranderingen in cortisol concentraties als gevolg van blootstelling aan een stress test. Twee eerdere studies vonden dat SNPs in *NR3C1* ((rs6195, rs41423247) geassocieerd zijn met de ontwikkeling van astma, maar resultaten van deze studies zijn nooit gerepliceerd. De rol die SNPs in *NR3C2* hebben in de ontwikkeling van astma is nooit eerder bestudeerd. In **hoofdstuk 6** is onderzocht wat de rol van SNPs in *NR3C2* en *NR3C1* is in de ontwikkeling van astma. Daarnaast is in deze studie onderzocht of het effect van SNPs in deze receptoren in de ontwikkeling van astma afhangt van de blootstelling aan perinatale stress. Resultaten van deze studie laten geen verband zien tussen SNPs in *NR3C2* en *NR3C1* en astma. Tevens zijn er geen aanwijzingen gevonden voor een interactie tussen SNPs in *NR3C2* en *NR3C1* en blootstelling aan perinatale stress. Dit suggereert dat genetische varianten in de mineralocorticoid en glucocorticoid receptor geen aantoonbare rol spelen in de ontwikkeling van astma.

Psychosociale stress is een risico factor voor het ontwikkelen van astma. Echter de mechanismen die hierbij een rol spelen zijn deels nog onduidelijk.

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CURRICULUM VITAE

Nienke Vink is geboren in Hengelo (Overijssel), Nederland, op 12 april 1979. Ze behaalde in 1997 haar VWO-diploma op de 'openbare scholengemeenschap de Waerdenborch' in Holten. Nadat ze 1 jaar Farmacie studeerde, startte ze in 1998 met haar studie Geneeskunde aan de Rijksuniversiteit van Groningen. In 2004 ontving ze haar artsenbul, waarna ze als arts-assistent Interne Geneeskunde, Cardiologie en Longziekten in het Medisch Centrum Leeuwarden en als keuringsarts bij Pharma-Bio-Research in Zuidlaren heeft gewerkt. In 2006 startte ze met haar studie Epidemiologie aan de Universiteit van Utrecht, hetgeen resulteerde in haar registratie als Epidemioloog A in 2008. In datzelfde jaar begon ze aan haar promotieonderzoek leidend tot dit proefschrift, gesuperviseerd door prof. dr. H.M. Boezen, prof. dr. J.G.M. Rosmalen en prof. dr. D.S. Postma. In de nabije toekomst zal ze worden geregistreerd als epidemioloog B.

Van 2006 tot en met 2010 werkte ze parttime als docent bij het Klinisch Trainingscentrum van het Universitair Medisch Centrum Groningen.

Nienke werkt momenteel bij de GGD Twente, waar ze in opleiding is tot arts infectieziektebestrijding.

TRAILS DISSERTATIONS

Sondeijker, F.E.P.L. (2006). Neuroendocrine and autonomic risk factors for disruptive behaviors in adolescents. Promotores: Prof. dr. F.C. Verhulst, Prof. dr. J. Ormel. Copromotor: Dr. R.F. Ferdinand.

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Dietrich, A. (2007). Autonomic nervous system function and behavioral characteristics in (pre)adolescents from a general population cohort. Promotores: Prof. dr. J. Neeleman, Prof. dr. J. Ormel. Copromotor: Dr. J.G.M. Rosmalen.

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Dijkstra, J.K. (2007). Status and affection among (pre)adolescents and their relation with antisocial and prosocial behavior. Promotor: Prof. dr. S. Lindenberg. Copromotor: Dr. R. Veenstra.

Amone, K.P. (2009). Examining the link between socio-economic position and mental health in early adolescents. Promotores: Prof. dr. J. Ormel, Prof. dr. A.J.Oldehinkel. Copromotor: Dr. H. Burger.

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Buschgens, C.J.M. (2010). It runs in the family – Early biological factors and family environment in children with ADHD symptoms. Promotores: Prof. dr. J.K. Buitelaar, Prof. dr. M.A.G. van Aken.

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